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Hypercholesterolemia and microvascular dysfunction: Altered mechanisms and interventional strategies

Phoebe Stapleton
West Virginia University

HYPERCHOLESTEROLEMIA AND MICROVASCULAR DYSFUNCTION:

ALTERED MECHANISMS AND INTERVENTIONAL STRATEGIES

PHOEBE STAPLETON

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Jefferson C. Frisbee, Ph.D., Chair
Robert W. Brock, Ph.D.
Stephen Alway, Ph.D.
Gregory M. Dick, Ph.D.
Alexandre d'Audiffret, M.D.

Department of Exercise Physiology
Center for Cardiovascular and Respiratory Sciences

Morgantown, West Virginia
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ABSTRACT

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Phoebe Stapleton

Cardiovascular disease has a number of risk factors and confounding variables associated with the development and progression of the condition. Hypercholesterolemia is one of the few independent risk factors of peripheral vascular disease (PVD) and coronary artery disease (CAD) with causes stemming from a genetic predisposition and lifestyle management. The primary symptom of these diseases can be described as an endothelial cell dysfunction, which hypercholesterolemia can exacerbate, leading to a pro-inflammatory state of elevated oxidant stress and significant reduction in nitric oxide (NO) bioavailability, a key mediator of endothelium-dependent dilation. This environment can culminate into an impairment of the vascular reactivity due to mechanical and metabolic alterations.

The mechanical microvascular remodeling is evident within dyslipidemia producing a decrease in vascular perfusion; however there are significant differences between the remodeling within the condition of hypercholesterolemia leading to an early evolution of MCP-1 and increased wall stiffness and thickness and later progression of hyperlipidemia leading to an increase in TNF-α and microvascular rarefaction.

While the reduction in NO bioavailability, due to a reduced production or oxidative scavenge, leads to a maintained yet altered mechanism of peripheral skeletal muscle arteriolar endothelium dependent dilation with a shift to the reliance on increased production of metabolites of arachidonic acid metabolism, via the cyclooxygenase and lipoxygenase enzymatic pathways. These metabolites include 12- and 15-lipoxygenase and prostacyclin, with strong dilator effects, in addition to, thromboxane A_2 a profound vasoconstrictor. The culmination of these products leads to a net reduced dilator effect evident in animals with genetic hypercholesterolemia.

Current interventions include a number of cholesterol lowering prescriptions and the inclusion of a regular exercise program, which have been shown to reduce cholesterol and improve the cardiovascular symptoms. Considerable attention has been given to the molecular mechanisms leading to the improvement of microvascular endothelial dysfunction and subsequent vascular reactivity; however the mechanistic consequences of these treatments are not well understood within the realms of inflammation, oxidative stress, and vascular reactivity. In both the hypercholesterolemic and normocholesterolemic groups the greatest benefit, with respect to inflammation and oxidant stress, was seen in the exercise only groups. Unexpectedly, the hypercholesterolemic groups saw no improvement in vascular reactivity to any of the interventions; while the normocholesterolemic group presented detrimental results to the vascular reactivity of the pharmaceutically treated animals. Therefore, the mechanistic and mechanical outcomes associated with the reported pleiotropic effects of the cholesterol lowering treatments warrants further study specific to hyper- and normocholesterolemic conditions.

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TABLE OF CONTENTS

LIST OF FIGURES

Chapter I

Chapter II

LIST OF TABLES

Introduction

While hypercholesterolemia, defined as excessively high plasma cholesterol levels, has emerged as a strong risk factor for cardiovascular disease (CVD). Data acquired by the National Health and Nutrition Examination Survey (NHANES) 2005–2006 found that the mean total serum cholesterol for Americans over the age of 20 was 199 mg/dl, approximating the American Heart Association (AHA) recommended level of 200 mg/dl (1). Unfortunately, 16% of adults were found to have total cholesterol levels of more than 240 mg/dl, a level considered by the AHA to carry twice the CVD risk of those individuals at the desired level (1; 55).

Total cholesterol can be broken down into a diagnostic lipoprotein profile, including high density lipoprotein (HDL), low density lipoprotein (LDL), intermediate density lipoproteins (IDL), very low density lipoprotein (VLDL), chylomicron remnants, and triglycerides. With respect to these markers, the AHA publishes recommendations summarized in Table 1 (1). HDL is considered to be beneficial as higher levels have been correlated with reduced risk of negative cardiovascular events, in large measure by promoting reverse cholesterol transport, an anti-atherogenic process resulting in cholesterol from peripheral tissues returning to the liver for subsequent processing (1). Elevated LDL cholesterol and triglycerides are considered detrimental as their increased concentration is well correlated with poor cardiovascular outcomes (1; 87). Current research has also suggested that IDL, VLDL, and chylomicron remnants may also play an active role in PVD and CAD development (87).

As high total cholesterol levels are considered to be a major independent risk factor for development of PVD and CAD, considerable attention has been directed toward evaluating the impact and mechanisms of cholesterol lowering therapies and interventions for cardiovascular outcomes (55; 87; 88). Cholesterol has been shown to interrupt and alter vascular structure,

mechanics, and function as it builds within the lining of the vascular wall, and can interfere with endothelial function leading to lesions, plaques, occlusion, and emboli; along with a reduction in healing, recovery, and appropriate management of ischemia/reperfusion injury (15; 35; 38; 45; 92). With specific relevance to the microcirculation, it has been clearly demonstrated that evolution of hypercholesterolemia is associated with endothelial cell dysfunction (8; 21; 35; 43; 93; 109). Additionally, reports have shown a near-complete abrogation in vascular nitric oxide (NO) bioavailability, elevated oxidant stress, and the creation of a strongly pro-inflammatory condition; symptoms which can culminate in profound impairments to vascular reactivity (8; 13; 27; 46; 77; 93; 95; 96; 103; 107). Investigation into vascular consequences of chronic hypercholesterolemia, the mechanisms through which these consequences occur, and the potentially beneficial effects of ameliorative therapies have received considerable attention in recent years (2; 4; 20; 27; 38; 77; 87; 91; 93).

Although a substantial risk factor for CVD, hypercholesterolemia has also been demonstrated to be one which can be manageable, as summarized in meta-analytic projects which have supported the use of pharmaceutical interventions to reduce cholesterol, with the outcome of lowering cardiovascular event incidence (4; 6). However, these studies have evaluated few vascular reactivity benefits associated independently of the overall cholesterol lowering benefit. Therefore, effective interventional treatment can be problematic, as the presence of specific genetic risk factors are frequently present. The condition of familial hypercholesterolemia (FH) is an inherited autosomal dominant disorder caused by variations to the low density lipoprotein receptor (LDLR) gene, preventing effective function and dramatically elevated levels of circulating LDL (52). While the phenotypic effects of the homozygous condition are more severe, the prevalence of the heterozygous condition affects approximately 1

in 500 individuals (25). Normally, LDL transports cholesterols and fats through the aqueous bloodstream to the cell surface where LDLR mediates its endocytosis, a process that is rendered ineffective in FH. A second inherited cause of hypercholesterolemia is familial combined hyperlipidemia (FCH), also known as type III hyperlipidemia, which presents high cholesterol and high triglyceride levels stemming from a number of gene polymorphisms (58). Interestingly, while the dyslipidemic profile of these two conditions differs, there is a striking similarity in the poor vascular outcomes (92).

Hypercholesterolemia and Vascular Dysfunction

The vascular endothelium, a single cell layer on the inner surface of all vessels, is capable of producing numerous bioactive molecules, thereby acting as an autocrine, paracrine, and endocrine organ (20). In a normal system, endothelial cells maintain vascular tone via endothelium-derived relaxing factors including NO, prostacyclin, and endothelium-derived hyperpolarizing factors (43) in an integrated balance with sympathetic and myogenic tone as well as parenchymal cell influences. These molecules help to regulate the homeostasis of the vascular system by adjusting to a number of systemic demands on blood flow, coagulation, inflammation, platelet aggregation, and signal transduction, with any decay in efficacy considered as dysfunction (26).

Nitric oxide (NO), a gas synthesized from the amino acid L-arginine through the enzyme nitric oxide synthase (NOS), has been widely considered as an endothelium-dependent regulator of vascular tone, with additional roles in preventing platelet activation, inhibiting oxidative stress, cell growth, and inflammation, among others (46; 66). Asymmetric dimethylarginine (ADMA) is an endogenous inhibitor of NOS through competitive inhibition with L-arginine (13). Given recent studies demonstrating an increased endogenous production of ADMA in

hypercholesterolemia and the inverse relationship between NO production and ADMA concentration, ADMA is currently under intensive evaluation as an additional risk factor for CVD (13; 103).

Previous studies within our laboratory and others have shown that dilator reactivity in response to NO-dependent stimuli is moderately impaired in hypercholesterolemic mice as compared to responses in control animals (36; 60; 90; 93; 96; 97; 110). This reduction is not due to an inability to react to the NO signal, as vessels are able to respond normally to NO donors, rather there is a reduction in the bioavailability of NO within vasculature either via deficits in production or due to increased oxidant scavenging (109). Additional data suggest that NO-mediated endothelium-dependent responses within a hypercholesterolemic milieu may differ between conduit vessels and the microcirculation, as peripheral resistance arterioles have a greater sensitivity to local metabolite production (29; 40; 105). Further, in hypercholesterolemic mice and diet induced hypercholesterolemic rabbits, compensatory mechanisms evolve to maintain endothelium-dependent dilation as a result of a decrease in NO bioavailability, and appear to involve altered patterns of arachidonic acid metabolism involving both the cyclooxyegnase and lipoxygenase pathways (2; 3; 21; 78; 93; 106). Arachidonic acid action within hypercholesterolemia is not limited to metabolite production inducing dilation, such as prostacyclin, which remains constant, but includes the production of thromboxane A_2 (TXA_2), a potent vasoconstrictor (27; 75). Hypercholesterolemic animals have shown a limitation to arachidonic acid induced dilation due to a counteractive increase in TXA_2 production during metabolism (15). While, similar hypercholesterolemic animals have shown an improvement in vascular reactivity and atherosclerotic lesions in animals who are thromboxane receptor deficient

(27; 49; 76). This mechanism may also lead to the reductions of endothelium dependent dilation seen in hypercholesterolemia.

The mechanical consequences of hypercholesterolemia and hyperlipidemia seem to differ, despite similar mechanistic outcomes. Upon evaluation of wall mechanics and microvessel density, the ApoE model has also been associated with profound microvessel rarefaction, marked by increases in TNF-α and thromboxane A_2 (TxA_2) while the LDLR mouse is portrays significant wall remodeling accompanied by an increase in monocyte chemotactic protein-1 (MCP-1) (92).

Hypercholesterolemia and Inflammation

Numerous studies have clearly established that hypercholesterolemia leads to an inflammatory response within the microvasculature, reflected by endothelial cell activation, leukocyte recruitment, rolling and adherence, as well as platelet activation and adhesion (84; 95). The decreased bioavailability of NO in hypercholesterolemia also diminishes the anti-inflammatory properties of the endothelial cell, permitting the activity of growth factors on the cell surface and platelet activation to act as chemoattractants to a parade of inflammatory events. Leukocytes begin to roll along the lumen and adhere to the cell wall, extravasate, and reside within the intimal space (13). Monocyte chemotactic protein-1 (MCP-1) and interleukin-8 (IL-8) have both been found to be important in hypercholesterolemic patients, acting to increase monocyte recruitment and adherence which leads to wall remodeling (15; 64; 67; 92). Macrophages, derived from monocytes, begin to accumulate LDL and oxidized LDL (oxLDL) which develop into foam cells between the basal lamina of the endothelium and the smooth muscle layer (20). These foam cells lead to the production of numerous inflammatory and

oxidative stress markers, cytokines, chemokines, and growth factors which aggravate the balance of endothelial equilibrium leading to vascular dysfunction.

Elevated cholesterol has also been shown to trigger the release of the inflammatory mediator C-reactive protein (CRP), a useful clinical marker of CVD (33; 98). It is hypothesized that CRP, via IL-6, may exacerbate vascular dysfunction by inhibiting eNOS, stimulating production of reactive oxygen species and increasing vascular permeability, and may also initiate the expression and stimulation of adhesion molecules, chemokine production, and thrombus formation within endothelial cells (67). Unfortunately, as a cellular marker of vascular inflammation, the source of CRP within the hypercholesterolemic condition is unclear (12).

Hypercholesterolemia and Oxidative Stress

Excess oxidative stress is caused by an imbalance between pro- and anti-oxidant enzymes, leading to an overproduction of free radicals, including superoxide, hydroxyl radicals, and lipid radicals, which may damage cellular components, interfering with normal function. Other molecules such as peroxynitrite, hydrogen peroxide, and hypochlorous acid are also oxidants, but are not considered free radicals. The two major sources of reactive oxidant species (ROS) within the vasculature are leukocytes (macrophages) recruited due to an endothelial injury signal and inefficiencies within smooth muscle cell mitochondrial metabolism (32).

Hypercholesterolemia may also increase activity of three major oxidant producing enzyme systems; NADPH oxidases (NOX), xanthine oxidase, and myeloperoxidase (MPO). NOX acts to transfer an electron to an oxygen molecule, forming superoxide or H_2O_2 (14). While seven NOX isoforms have been identified (NOX1-5, DUOX 1 and 2), four of these (NOX1, 2, 4, and 5) have been recognized within the vascular wall, with NOX2 responsible for the greatest impact on ROS-related decreases to NO bioavailability (85). Xanthine oxidase forms superoxide

and H_2O_2 during the reduction of oxygen, while myeloperoxidase is produced by neutrophils and monocytes and produces a toxic hypochlorous acid; within a pathological condition overactive enzymes can lead to the overproduction these radicals, leading to scavenging of NO molecules, uncoupling of eNOS, and/or the formation of peroxynitrite (32). MPO is an enzyme produced by neutrophils and monocytes using hydrogen peroxide to produce hypochlorous acid. Additionally, eNOS uncoupling and substrate reduction (tetrahydrobiopterin (BH_4) and L-arginine), can transform eNOS into a superoxide generating enzyme which can, in turn, produce greater amounts of oxidant radicals and hydrogen peroxide in addition to NO production (23; 66).

Evaluating the nitrotyrosine levels within the system will give insight to the amount of peroxynitrite formation, reductions in NO bioavailability, and the nitrosylation of protein tyrosines leading to oxidant radical formation and susbsequent oxidation. However, cholortyrosine will indicate MPO activity. While, evaluating the concentration of isoprostanes will indicate the amount of lipid peroxidation within the system; this is a systemic measurement and the molecules measure are not directly responsible for the endothelial cell activation as they are freely circulating throughout the blood, but may indicate a propensity of lipid peroxidation within the system.

A range of antioxidant mechanisms are in place to minimize the balance of the effects of ROS, including superoxide dismutase (SOD), glutathione peroxidase (GPx4), catalase, and thioredoxin reductase. SOD, which comes in three forms, soluble cytoplasmic (SOD1), extracellular (SOD3) containing copper and zinc and mitochondrial (SOD2), containing manganese. SOD2 is the main cellular antioxidant system in all cell types is capable of converting superoxide radicals to H_2O_2 and oxygen (98). GPx4 reduces H_2O_2 and lipid peroxides to water and lipid alcohols, and reduces the development of atherosclerosis during

hypercholesterolemia through the inhibition of lipid peroxidation and a decreased sensitivity of endothelial cells to oxidized lipids (31). Catalase acts to reduce hydrogen peroxide to oxygen molecules and water. Within the pathological state of hypercholesterolemia, antioxidant systems are unable to handle the increased demand and the ROS production exceeds capacity. Thioredoxin reductase acts to enhance other antioxidant activity by recycling reducing agents.

Within hypercholesterolemia, reactions between radicals and lipoproteins can lead to production of lipid radicals or oxLDL which can accumulate within the cellular membrane, disrupting normal cellular function and ultimately leading to poor vascular function (11; 43). The increased lipid radicals within hypercholesterolemia can, impact endothelium-dependent dilation through a reduced bioavailability of NO (11). This conclusion is further supported with evidence that cholesterol fed animals treated with polyethylene-glycolated–SOD demonstrate an improved endothelium dependent dilation, while normocholesterolemic animals did not show any effects (63). OxLDL can interact directly with the endothelial cell through interactions with the lectin-like oxLDL receptor (LOX-1), an endothelial receptor for oxidized LDL in endothelial cells (83). This receptor is induced by a variety of inflammatory cytokines, oxidative stress, hemodynamic changes, and abundance of ox-LDL (83). Interactions with oxLDL causes further injury to the endothelial cell subsequently activating platelets signaling a variety of adhesion and inflammatory molecules including MCP-1. In addition to oxLDL, LOX-1 can bind advanced glycation end products (AGE), activated platelets, and leukocytes all furthering inflammatory and oxidative processes (83).

Hypercholesterolemia and Pharmaceutical Therapies

Statins, 3-hydroxy-3-methylglutaryl-coenzyme A (HMG CoA) reductase inhibitors, are currently one of the most widely prescribed drugs on the market today. They target liver HMG

CoA reductase activity and inhibit the production of a cholesterol precursor, mevalonic acid. They also specifically act to change the conformation of HMG-CoA reductase when bound, preventing a functional structure (91). This enzymatic inhibition acts to prevent protease activation of sterol regulatory element binding proteins (SREBPs) from the endoplasmic reticulum, thereby preventing nuclear translocation and upregulation of LDL gene expression, limiting hepatic cholesterol production (91).

Statins have been identified to have numerous positive outcomes associated with their direct cholesterol lowering (48; 54; 56; 69). In addition to these, vasculoprotective properties such as increased NO bioavailability, antioxidant, anti-inflammatory and immunomodulatory properties leading to an overall improvement of endothelial function have also been identified; yet specifically identifying the discrete result in human hypercholesterolemic patients is difficult as the cholesterol lowering benefits are similar (41; 102). These diverse positive vascular outcomes are most easily identified while using a genetically modified murine model, as the lipid-lowering results become null, leaving the pleiotropic effects evident. While similar, these consequences independent of the cholesterol lowering effects include, but are not limited to, reducing inflammation, decreases in ROS, increases in NO bioavailability and endothelial function, decreases in platelet activation and aggregation, reduction in coagulation and decreases in cellular proliferation, among others (106). Additionally, statin therapy has been found to significantly improve endothelial function (based on flow-mediated dilator responses) in hypercholesterolemic patients who had also been diagnosed with PVD (30). While this beneficial effect may have resulted from an increased NO bioavailability, the underlying mechanisms have not been fully understood (51).

Ezetimibe (Zetia) is a selective agent which acts to prevent cholesterol absorption in the intestine through targeting Niemann-Pick C1-like 1 protein (NPC1L1), which is expressed on the intestinal cell surface and is a transporter with secretion signal and sterol-sensing domains. Ezetimibe will inhibit this protein, thereby blocking LDL uptake from the intestine (61; 62). The subsequent reduction in cholesterol transport to the liver stimulates a compensatory increase in LDLR expression, thereby increasing vascular clearance with no serious side effects (38). While cholesterol lowering therapies have shown a positive correlation with reductions in cardiovascular events, ezetimibe has recently begun to show pleiotropic effects such as reductions in liver lipids, reductions in lipid lesions, reductions in ADMA levels, and increases in eNOS mRNA expression (20; 69).

When used in combination, ezetimibe and statins (e.g., Vytorin) act via complementary pathways to prevent cholesterol absorption from the intestine and hepatic production. Long term co-administration of these drugs have been shown to reduce LDL blood cholesterol levels by 60% while concurrently raising HDL levels and limiting liver toxicity, myotoxicity and/or rhabdomyolysis traditionally caused by statin treatment alone (17; 38; 82). However, at present, the side effects of the combined therapy are not well described, and it is unclear how effective these are for impacting the inflammatory and oxidative stress profiles (54; 56; 89). This class of drugs can only partially impact the cholesterol levels of patients diagnosed with familial hypercholesterolemia, as these patients lack the LDL receptor necessary for the uptake of the pharmaceutically reduced endogenous production and intestinal uptake of the available LDL.

Oxidant Stress, Inflammation and Pharmaceutical Therapies

While lowering overall cholesterol levels can lead to a decrease in vascular oxidative stress and thereby improve endothelial function, some groups have found antioxidant properties

to be a pleiotropic effect of statins (100). When evaluated to examine NO in a biologically active form, cholesterol lowering drugs were shown to increase the efficiency of the NOS system, while simultaneously showing an inactivation of oxygen radicals within the system (44). These drugs may not act directly upon the radicals, but instead act to reduce oxidant stress by decreasing substrate availability for these radicals to act upon or by increasing antioxidant enzymatic activities, such as SOD (37). Statins have found to act upon the p21 Rac protein interrupting the NOX subunit assembly working directly to inhibit the production mechanism of superoxides through disruption of the NOX enzyme (104). Some studies have shown positive results with respect to lipid peroxidation, including the increase of an antioxidant effect leading to a decrease in ox-LDL with combination ezetimibe/statin treatment (39).

Pharmaceutical treatments have been shown to influence inflammation through the decrease of systemic markers of inflammation and to increase the stability of existing plaques, thereby reducing the risk for thrombosis. Some groups are considering treating LDL as means to managing inflammation and preventing atherosclerotic lesions with mixed reviews and results (101; 111).

CRP has been commonly used as a marker of inflammation in a clinical setting since it is associated with myocardial infarction and low-grade cardiovascular inflammation. Statin drugs have been shown to decrease CRP in numerous human studies, including JUPITER, ENHANCE, CARE, and PRINCE, regardless of their lipid lowering effects (81). Additional studies have shown an interference with the inflammatory process, impacting the expression of interleukins, adhesion molecules, platelet aggregation, and chemoattractants including IL-1, IL-6, IL-8, NF-κB, and TNF-α culminating in the decrease of CRP (50).

Animal studies have shown atorvastatin to reduce inflammatory markers such as MCP-1 and the activation of the nuclear factor NF-κB (10). More recently, as the pleiotropic effects of these interventions are being evaluated, some studies have found reductions in the adhesion molecules ICAM, VCAM, E-selectin, P-selectin, MMP-9, and platelet aggregation. These reductions are leading some to the conclusion that pharmaceutical therapies may reduce or limit the formation and instability of atherosclerotic plaques (16).

Hypercholesterolemia and Exercise

The AHA and American College of Sports Medicine (ACSM) have recently released joint guidelines recommending aerobic and resistance physical activities for individuals under the age of 65 to maintain health, reduce risk of chronic disease states, and manage current risk factors including hypercholesterolemia (34; 47; 94). Hypercholesterolemia has been shown to impair aerobic capacity by impairing dilator regulation, thought to be due to a lack of vascular reactivity stemming from a reduction in NO bioavailability (57). However, this decline in vascular reactivity may also be due to wall remodeling as seen in the LDLR mouse model of FH or poor blood flow distribution due to microvessel rarefaction seen in the ApoE mouse model of FCH (92). These may lead to a decrease in oxygen transport to working skeletal muscles during the hyperemic demand of exercise, further reducing aerobic capacity (57).

Few groups examine dose-response relationships between exercise training and cholesterol adaptations. Some have suggested that exercise can alter blood lipids at low training volumes, although effects may not be significant until certain caloric thresholds are met. Exercise training has rarely been shown to have a direct effect on total cholesterol or LDL levels; however, significant increases in HDL and decreases in triglycerides have been identified (22). This may be a function of activity intensity, as a 1200 – 2200 kcal/week exercise program

performed at moderate intensities has been shown to reduce total and LDL cholesterol levels (22).

A number of moderate-intensity exercise programs have shown improvements to systemic aerobic capacity, effectively reversing early stage hypercholesterolemic changes within the vasculature, including improved vascular reactivity, NO bioavailability and eNOS activity (28; 105). These increases in NO bioavailability in humans and animal models of hypercholesterolemia have been attributed to eNOS expression and production of NO, due to a chronic rise in shear stress with exercise, as opposed to an increase in SOD or reduction in oxidant stress (19). Exercise and shear stress have also been shown to improve mechanisms of endothelial vasodilation other than NO, such as prostaglandin release (93). Exercise has also been shown to ameliorate increases in inflammatory and oxidative stress markers during chronic disease state, which would benefit many low-grade inflammatory conditions (5).

Oxidant Stress, Inflammation, and Exercise

Cellular respiration and metabolism are directly linked to physical activity and exercise as they are the source responsible for muscle action. In the presence of oxygen, aerobic respiration allows for the production of ATP, where glucose is broken down to pyruvate and enters the mitochondria for further processing via Kreb's cycle and oxidation via the electron transport chain. Unfortunately, minor inefficiencies within the mitochondria, including leaky membranes and limited cofactor availability, lead to a reduced ATP generation and the excess buildup of oxidants (80).

In acute exercise alterations to the mitochondrial electron transport chain is a direct source of oxidant stress due to the significant amount of oxidative handling throughout the system (50). Therefore, any inefficiencies associated within this system are multiplied as

mitochondrial requirements increase due to an increase in activity, specifically during acute exercise when there is an increase in whole body oxygen consumption thereby increasing the generation of ROS by active tissues (7). During the production of these mitochondrial-derived radicals, there is also an increase of the pro-oxidant enzymes xanthine oxidase, myeloperoxidase, and NOX (42). The upregulation of these enzymes causes an increase in plasma markers of ROS, such as F_2-isoprostanes (9). This increased oxidant stress, while promoting negative cardiovascular effects, has recently been shown to occur in conjunction with increases in antibodies to ox-LDL and antioxidant enzymes (catalase) after one week of activity in mice (59). These changes suggest that after only a week of moderate activity, there is an initiation to improve hypercholesterolemia, limit the progression of foam cell development, and increase antioxidant enzyme activity within exercising and sedentary states. As exercise persists, mitochondrial and antioxidant enzymes also improve; specifically, an increase in expression of Cu/Zn superoxide dismutase (SOD-1) and glutathione peroxidase lead to a higher oxidant handling capacity and contribution to improved function (19; 61). As a consequence, there is a decrease in the plasma markers of oxidative stress F2-isoprostane, myeloperoxidase, and malondialdehyde (86). Exercise training has also been shown to have a direct positive effect on the induction of eNOS and ecSOD (endothelial cell SOD), potent antioxidants. These increases are interdependent, as $eNOS^{-/-}$ mice seem to be unaffected an increase in ecSOD (24).

Exercise and increases in NO have also been shown to induce HO-1 (heme oxygenase-1) expression. HO-1 products have similar anti-oxidant and anti-inflammatory effects, in addition to the inhibition of NF-KB an oxidant stress sensitive transcription factor (99). The inhibition of NF-kB leads to a decrease of the entire downstream signaling cascade, which could be the link to many of the NO-mediated anti-inflammatory effects observed with chronic exercise such as

decreases in leukocyte binding, chemotaxis, aggregation of platelets, and proliferation of smooth muscle cells (18).

In the past, inflammation associated with physical activity has been described as the reaction to a number of repeated micro-traumas to the muscle (68). However, muscle has recently been identified as an endocrine organ, possessing the ability to manufacture and release humoral mediators directly into the system in response to muscle contraction (72). This establishes a link between skeletal muscle activity and anti-inflammatory effects (70). The cytokines produced, identified as myokines, include IL-6, IL-8, IL-15, brain-derived neurotrophic factor (BDNF), leukemia inhibitory factor (LIF) FGF21 and follistatin-like-1: each are regulated in some manner by the contraction or contractility of muscle (71). With respect to IL-6, the myokine hypothesis suggests that both type I and type II muscle fibers are capable of producing and releasing IL-6, which may act locally through AMPK signaling or systemically to improve hepatic glucose production and lipid metabolism (73).

During acute exercise, there is an immediate increase in a variety of anti-inflammatory cytokines, such as IL-6, IL-1ra, sTNFR (soluble TNF-α receptor), and IL-10. However, pro-inflammatory cytokines TNF-α (tumor necrosis factor- α) and IL-1 are generally not changed (74). Chronic exercise leads to a reduction of systemic and local markers of inflammation within the vasculature has been well established within the literature (79). As exercise persists to a chronic state pro-inflammatory markers CRP, TNF-α, IFN-γ, MCP-1, IL-6, IL-8, and MMP-9 have all been shown to decrease from initial baseline levels; whereas anti-inflammatory markers IL-10 and TGF-β increase indicating the development of a less inflammatory phenotype (53; 65). The timeline and exact mechanisms by which a chronic increase in activity will lead to modest

improvements in low-grade inflammation are uncertain (108). However, some groups are focusing on the "long-term anti-inflammatory effects of exercise" (65; 70).

Conclusion

Given the severity of hypercholesterolemia as a risk factor for the progression of negative CVD outcomes, the pathways of effective interventional strategies to manage cholesterol levels, improve vascular reactivity, and restore NO bioavailability warrant continued investment. Pharmaceutical therapies have presented a variety of vasculoprotective effects which are not fully understood, but involve a complex interaction between vascular signaling mechanisms, oxidant stress and chronic inflammation. Additionally, physical activity and exercise have long been suggested as means to modify CVD and manage cholesterol. Current evidence also supports the theory of a long term anti-inflammatory effects through modifications of the IL-6 and CRP pathways, along with anti-oxidative effects of increased anti-oxidant enzyme expression and activity leading to a higher oxidant handling capacity at rest and during activity. These data suggest that the pleiotropic effects of exercise and conventional pharmaceutical therapies may be most beneficial when used in combination.

Therefore, the purpose of this study was to investigate the condition of hypercholesterolemia and how it relates to mechanical adaptations to vascular reactivity, the altered mechanisms of endothelium dependent dilation, and the consequences of current clinically-relevant ameliorative therapies.

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DIFFERENTIAL IMPACT OF FAMILIAL HYPERCHOLESTEROLEMIA AND COMBINED HYPERLIPIDEMIA ON VASCULAR WALL AND NETWORK REMODELING IN MICE

Phoebe A. Stapleton[1,2], Adam G. Goodwill[1,3], Milinda E. James[1,3],
Alexandre C. d'Audiffret[1,4] and Jefferson C. Frisbee[1,3]

Center for Cardiovascular and Respiratory Sciences[1], Division of Exercise Physiology[2], Department of Physiology and Pharmacology[3], and Division of Vascular and Endovascular Surgery[4]
West Virginia University School of Medicine, Morgantown, WV 26506

Running Head: Dyslipidemia and vascular remodeling

ABSTRACT

Genetic familial hypercholesterolemia (FH) and combined hyperlipidemia (FCH) are characterized by elevated plasma LDL (FH) and LDL/triglycerides (FCH), with mouse models represented by LDL receptor (LDLR) and apolipoprotein E (ApoE) gene deletion mice, respectively. Given the impact of FH and FCH on health outcomes, we determined the impact of FH/FCH on vascular structure in LDLR and ApoE mice. LDLR, ApoE and control mice were utilized at 12-13 and 22-23 weeks when gracilis arteries were studied for wall mechanics and gastrocnemius muscles were harvested for microvessel density measurements. Conduit arteries and plasma samples were harvested for biochemical analyses. Arteries from ApoE and LDLR exhibited blunted expansion versus control, reduced distensibility and left-shifted stress versus strain relation (LDLR>ApoE). Microvessel density was reduced in ApoE and LDLR (ApoE>LDLR). Secondary analyses suggested that wall remodeling in LDLR was associated with cholesterol and MCP-1, while rarefaction in ApoE was associated with TNF-α, triglycerides and vascular production of TxA_2. Remodeling in ApoE and LDLR appears distinct; as that in LDLR is preferential for vascular walls, while that for ApoE is stronger for rarefaction. Remodeling in LDLR may be associated with cellular adhesion, while that in ApoE may be associated with pro-apoptotsis and constrictor prostanoid generation.

INTRODUCTION

Numerous previous studies have determined that severe dyslipidemia, including both hypercholesterolemia and hypertriglyceridemia represent significant risk factors for the development of peripheral artery diseases and negative health outcomes (10, 13, 15, 23, 31). Overwhelmingly, these address alterations to patterns of atherosclerotic plaque, lesion and fatty streak depositions within the vascular lumen, the propensity for the creation of emboli, the contribution to occlusive disease states, and downstream tissue/organ outcomes (3, 15, 20, 39). While unquestionably of considerable clinical significance, these overt pathological states do not provide a complete understanding of the alterations to vascular structure and function that accompany dyslipidemia.

We have recently determined that in two mouse models of genetic dyslipidemia on the C57/Bl6/J background, the low density lipoprotein receptor gene deletion (B6.129S7-*Ldlr*^{tm1Her}/J; LDLR) and the apolipoprotein E gene deletion (B6.129P2-*Apoe*^{tm1Unc}/J; ApoE) the patterns of endothelium-dependent arteriolar reactivity are significantly altered from their control strains (C57; refs: 18, 40). In terms of clinical relevance, the LDLR mouse represents a model of familial hypercholesterolemia (FH; refs: 27, 37), characterized by striking elevations in plasma LDL levels, while the ApoE mouse represents familial combined hyperlipidemia (FCH; refs: 35, 36, 45), characterized by elevated plasma LDL (although not to levels in FH) and triglycerides. Specifically, both the LDLR and ApoE mouse models exhibit a striking loss of vascular nitric oxide bioavailability, as demonstrated by vascular responses to nitric oxide-dependent stimuli (11, 40, 41, 44). However, this is more complicated than a simple loss of NO bioavailability. Previous studies in our laboratory (40) and by others (1) have suggested that this loss of NO bioavailability may be partially compensated for through alterations to arachidonic acid metabolism via lipoxygenases. Further, other reports exist suggesting that there may also be an additional shift in arachidonic acid metabolism with hypercholesterolemia, increasing the production of thromboxane A_2 (TxA_2) versus prostacyclin (PGI_2) which also has the potential to negatively impact vascular function (18, 25). Finally, taken in combination with recent work from Wolfle and de Wit, suggesting that conducted responses in microvessels of LDLR mice were largely intact (43), and from Pfister *et*

al. indicating an alteration in arachidonic acid metabolism in vascular tissue from hypercholesterolemic rabbits (34), and it becomes clear that the net vascular outcomes from dyslipidemia can be very complicated.

From the perspective of vascular outcomes and the determination of tissue perfusion, one area that requires further attention is that of the vascular remodeling that accompanies genetic dyslipidemia, and this should incorporate two different elements of remodeling; vascular wall mechanics and microvessel network structure. While studies exist which describe wall stiffening with genetic dyslipidemia (2, 7, 16), these are limited by the analyses utilized and do not provide for a more thorough understanding. Additionally, the study of microvessel density under conditions of dyslipidemia has been extremely limited, although what evidence is available suggests that this may be reduced with hypercholesterolemia (9, 24).

The purpose of the present study was to determine differences in skeletal muscle microvascular remodeling (i.e., resistance artery wall mechanics and microvessel density) under conditions of FH and FCH utilizing the LDLR and ApoE mouse models of these conditions. The current study was designed to test the hypothesis that the progression of FH and FCH, while causing a remodeling of the skeletal muscle vasculature in both conditions, will be distinct in their vascular outcomes owing to differences in lipid and inflammatory profiles and impacts on endothelial function. This study will not only provide information regarding relevant changes to two key indices of vascular structure, it will also evaluate the temporal development of these alterations, involving mice at 12-13 and 22-23 weeks of age. Finally, these results are compared with an extensive analysis of biomarkers between the three strains to determine if differences in outcomes may be associated with a specific clustering of contributing elements. It is believed that this process may provide a framework for targeted interventional procedures to ameliorate the poor vascular outcomes associated with genetic dyslipidemia.

MATERIALS AND METHODS

Animals: This study utilized three strains of mice; C57/Bl6/J as the controls, and the LDLR and ApoE mice as models of FH and FCH, respectively. All mice were purchased from Jackson Laboratories (Bar Harbor, ME). Male mice of each strain were fed standard chow and drinking water *ad libitum* and were housed in the animal care

facility at the West Virginia University Health Sciences Center and all protocols received prior IACUC approval. At 12-13 or 22-23 weeks of age, mice were anesthetized with injections of sodium pentobarbital (50 $mg \bullet kg^{-1}$ i.p.), and received tracheal intubation to facilitate maintenance of a patent airway. In all mice, a carotid artery was cannulated for determination of arterial pressure. After surgical removal of gracilis muscle resistance arteries (see below), deeply anesthetized mice received a bilateral pneumothoracotomy followed by cardiac puncture, wherein venous blood aliquots were drawn into tubes containing either heparin (for lipid determination) or K_2-EDTA (for all other analyses). Samples were immediately frozen in liquid N_2 and processed as batches for lipid profiles (Wako) and inflammatory markers (Millipore) using commercially available kits.

Preparation of Isolated Skeletal Muscle Resistance Arterioles: In anesthetized mice, the right gracilis artery was removed, cannulated, and extended to its approximate *in situ* length (14). Following equilibration, the perfusate and superfusate were replaced with Ca^{2+}-free physiological salt solution and vessels were treated with 10^{-7} M phenylephrine until all tone was abolished. Subsequently, intralumenal pressure was altered, in 20 mmHg increments, between 0 mmHg and 160 mmHg and the inner and outer diameter of arteries was determined at each pressure. To ensure that a negative intralumenal pressure was not exerted on the vessel, 5 mmHg was used as the "0 mmHg" intralumenal pressure point; all other values of intralumenal pressure were multiples of 20 mmHg up to 160 mmHg. Specific pressures were randomized to prevent the occurrence of ordering effects. After 10 minutes at each intralumenal pressure, the inner and outer diameter of the passive arteriole was determined. These data were used to calculate arteriolar wall mechanics which were used as indicators of structural alterations to individual microvessels (19). Vesse wall thickness was calculated as:

$$WT = \frac{(OD - ID)}{2}$$

where WT represents wall thickness (μm) and OD and ID represent arteriolar outer and inner diameter, respectively (μm). Arteriolar cross-sectional wall area (CSWA; μm^2), assuming the arteriole is cross-sectionally round, is calculated as:

$$CWSA = \left[\pi\left(OD/2\right)^2\right] - \left[\pi\left(ID/2\right)^2\right]$$

Incremental arteriolar distensibility ($DIST_{INC}$; % change in arteriolar diameter/mmHg) was calculated as:

$$DIST_{INC} = \left(\Delta ID \Big/ \left(ID_i \times \Delta P_{IL} \right) \right) \times 100$$

where ΔID represents the change in internal arteriolar diameter for each incremental change in intralumenal pressure (ΔP_{IL}), and ID_i represents the initial internal diameter prior to the pressure change.

Circumferential wall stress is a measure of the average amount of distending force (in this case pressure; expressed in N/m^2) exerted on a deformable object. For the calculation of circumferential stress, intralumenal pressure was converted from mmHg to N/m^2, where 1 mmHg=1.334×10^2 N/m^2. Circumferential stress (σ) was then calculated as:

$$\sigma = \left(P_{IL} \times ID \right) \Big/ \left(2WT \right)$$

Circumferential strain is a measurement of the degree of deformation exhibited by an object (in this case the vessel wall) as a result of the imposed stress. Circumferential strain (ε) was calculated as:

$$\varepsilon = \left(ID - ID_5 \right) \Big/ ID_5$$

where ID_5 represents the internal arteriolar diameter at the lowest intralumenal pressure (i.e., 5 mmHg).

The tangential elastic modulus (E_T) describes an object's tendency to be deformed elastically in response to an applied stress. A vessel's E_T is defined as the slope of its circumferential stress versus strain relation. To determine tangential elastic modulus (E_T), the stress versus strain curves from each vessel were fit (OLS, $r^2>0.85$) with the following exponential equation:

$$\sigma = \sigma_5 \, e^{\beta \varepsilon}$$

where σ_5 represents circumferential stress at ID_5 and β is the slope coefficient, which will be a function of the independent variable, ε. E_T was then estimated at different values of circumferential stress from the derivative of the exponential curve:

$$E_T = d\sigma / d\varepsilon = \beta\sigma_5 e^{\beta\varepsilon}$$

Determination of Microvessel Density: While under anesthesia, the right gastrocnemius muscle from each mouse was removed, rinsed in physiological salt solution and lightly fixed in 0.25% formalin. Muscles were embedded in paraffin and cut into 5 μm cross sections, which were stained with *Griffonia simplicifolia* I lectin (Sigma), and mounted on microscope slides, as described previously (14). Using epifluorescence microscopy, localization of labeled microvessels was performed with a Nikon E600 upright microscope with a 20x objective lens (Plan Fluo phase NA 0.5). Excitation was provided by a 75 watt Xenon Arc lamp through a Lambda 10-2 optical filter changer (Sutter Instrument Company, Novato, CA) controlling a 595nm excitation filter and a 615 nm emission filter. All acquired images from individual sections were analyzed for number of microvessels and number of skeletal muscle fibers using commercially available software.

Measurement of Vascular Nitric Oxide Bioavailability: From each mouse, the aorta was removed and vascular nitric oxide production was assessed using amperometric sensors (World Precision Instruments). Briefly, aortae were sectioned longitudinally, pinned in a silastic coated dish and superfused with warmed (37°C) physiological salt solution equilibrated with 95% O_2/5% CO_2. The nitric oxide sensor (ISO-NOPF 100) was placed in close apposition to the endothelial surface and a baseline level of current was obtained. Subsequently, aortae were exposed to acute challenge with methacholine (10^{-6} M) and changes in current were determined. To verify that recorded data represented nitric oxide release, responses were re-evaluated following acute treatment of aortae with L-NAME (10^{-4} M).

Determination of Vascular Metabolites of Arachidonic Acid: Vascular production of 6-keto-prostaglandin $F_{1\alpha}$ (6-keto-$PGF_{1\alpha}$; the stable breakdown product of PGI_2; ref. 32), and 11-dehydro-thromboxane B_2 (11-dehydro-TxB_2; the stable breakdown product of TxA_2; ref. 8) in response to challenge with arachidonic acid within the three mouse strains was assessed using pooled conduit arteries (femoral, saphenous, iliac, carotid arteries) from each mouse. Vessels were incubated in microcentrifuge tubes in 1 ml of physiological salt solution for 30 minutes under control conditions (21% O_2), after which time arachidonic acid (10^{-5} M) was added to the tube for an additional 30 minutes.

After the second 30 minute period, the solution was transferred to a new tube, frozen in liquid N_2 and stored at -80°C. Metabolite release by the vessels was determined using commercially available kits for 6-keto-$PGF_{1\alpha}$ and 11-dehydro-TxB_2 (Cayman).

Statistical Analyses: All data are presented as mean±SEM. Differences in passive mechanical characteristics and microvessel density between mouse strains were assessed using analysis of variance (ANOVA) or regression analyses, with Student-Newman-Keuls-test post-hoc, as appropriate. Data describing tangential elastic modulus versus stress relations were fit with semi-logarithmic regression equations, each of which provided a significant F-statistic for the regression and an adjusted $r^2>0.90$. Differences between regression coefficients were evaluated using ANOVA with post-hoc tests, as appropriate. In all cases, $p<0.05$ was taken to reflect statistical significance.

RESULTS

At the time of use, body mass was not different between the three strains of mice at either the 12-13 week or 22-23 week ages. At 12-13 weeks, C57 averaged 22±2 g, while ApoE and LDLR averaged 21±2 and 22±2 g, respectively. At 22-23 weeks, C57 averaged 31±2 g, as compared to 30±3 g for ApoE and 31±2 g for LDLR. While no differences were observed for mean arterial pressure across the three strains at 12-13 weeks (84±6 mmHg for C57; 86±5 mmHg for ApoE and 90±5 mmHg for LDLR), LDLR mice exhibited a significant increase in blood pressure by 22-23 weeks, reaching 112±6 mmHg, as compared to 84±5 mmHg in C57 and 92±5 mmHg in ApoE.

Figure 1 presents the severity of hypercholesterolemia (Panel A) and hypertriglyceridemia (Panel B) across the three mouse strains in the present study. While both ApoE and LDLR were significantly hypercholesterolemic as compared to C57 at both 12-13 and 22-23 weeks of age, total cholesterol was also significantly elevated in LDLR as compared to ApoE at both ages (Panel A). In contrast, plasma triglyceride levels were only elevated in ApoE mice, and this effect was present at both age ranges (Panel B). Between age groups, within a strain, there were no differences in plasma cholesterol or triglycerides.

Figure 2 summarizes data describing basic deformational alterations to skeletal muscle arteries of C57, ApoE and LDLR mice at 12-13 and 22-23 weeks of age in response to increasing intralumenal pressure. The increase in arterial inner (Panel A) and outer diameter (Panel B) in response to increasing intralumenal pressure was

blunted in ApoE and LDLR as compared to responses in C57. This blunting was greater with regard to inner diameter in LDLR mice, and as such there was a tendency for wall thickness to be elevated in LDLR with increasing age (Panel C). Total cross-sectional wall area demonstrated some decline with age within a strain, but with the exception of comparisons to the younger C57 mice, this did not exhibit a consistent pattern (Panel D). The tendency for an increased wall thickness with increasing hypercholesterolemia was also evident in terms of wall:lumen ratio (Panel E), as the older group of LDLR exhibited a significantly greater ratio over the range of intralumenal pressure. Finally, arterial wall incremental distensibility was reduced in LDLR with age as compared to either ApoE or C57 mice throughout the early range of intralumenal pressures (Panel F). However, beyond these points, all of the curves became superimposable.

Figure 3 presents data describing the changes in arterial circumferential wall stress versus strain (Panel A) and the tangential elastic modulus versus stress (Panel B) relations across the three strains. With increasing age and dyslipidemia, there was a progressive left-shifting of the stress versus strain relation that was most pronounced in LDLR as compared to ApoE (where no statistically significant shift in the curve was determined). This was also apparent in the E_T versus stress relation, where a modest trend toward an increasing slope in arteries from ApoE versus C57 was present, but that a striking shift in this relation in arteries from LDLR was evident compared to either other group.

The changes in microvessel density with age in control and dyslipidemic mice are summarized in Figure 4. While microvessel density was stable in skeletal muscle of C57 mice between 12-13 and 22-23 weeks of age, a consistent and increasing rarefaction in muscle of ApoE mice was exhibited, such that it was reduced at 12-13 weeks and a further rarefaction continued to 22-23 weeks of age. In contrast, there was no evidence for a significant rarefaction of the skeletal muscle microcirculation of LDLR at 12-13 weeks of age. However, the data presented in Figure 4 suggest that microvascular rarefaction was delayed in LDLR as compared to ApoE, as a significant reduction in microvessel density was evident by 22-23 weeks of age.

Data describing the plasma levels of specific biomarkers or contributors to vascular dysfunction are summarized in Figure 5. Plasma nitrotyrosine, an indicator of chronic vascular oxidant stress, was significantly

elevated in both ApoE and LDLR as compared to C57, and this was evident at both age ranges (Panel A). However, no differences were determined between ApoE and LDLR in terms of nitrotyrosine. Plasma concentrations of tumor necrosis factors-α (TNF-α) were elevated in both ApoE and LDLR as compared to C57 at both age ranges, although the magnitude of this increase was greater in ApoE mice (Panel B). In both ApoE and LDLR, plasma interleukin-1β (IL-1β) was elevated as compared to levels determined in C57 at both 12-13 and 22-23 weeks of age, although no differences were determined between the two dyslipidemic strains (Panel C). Panel D presents plasma levels of monocyte chemoattractant protein-1 (MCP-1) between the three strains. While ApoE mice consistently demonstrated a significant increase in MCP-1 versus C57, this effect was much more pronounced in LDLR, where MCP-1 levels were also increased over that in ApoE.

Using conduit arteries, indices of vascular endothelial function are summarized in Figure 6. Following challenge with arachidonic acid, pooled arteries from all strains demonstrated a comparable, maintained ability to produce PGI_2, as estimated from its breakdown product, 6-keto-$PGF_{1\alpha}$ (Panel A). In contrast, arachidonic acid-induced production of TxA_2, estimated from its breakdown product 11-dehydro-TxB_2, was significantly increased at both 12-13 and 22-23 weeks of age in arteries from ApoE as compared to C57 mice (Panel B). A similar pattern was also evident for thromboxane production in arteries from LDLR mice, although this lagged behind that in ApoE. Finally, vascular bioavailability of nitric oxide, estimated from its production following challenge with methacholine was dramatically attenuated in arteries from both ApoE and LDLR versus control at both 12-13 and 22-23 weeks (Panel C). Treatment of arteries with L-NAME abolished methacholine-induced nitric oxide production in all cases (data not shown).

DISCUSSION

While previous studies involving dyslipidemic humans and animal have demonstrated that elevated plasma cholesterol and triglycerides represent a significant risk factor for the development of atherosclerotic vascular disease (10, 13, 15, 23, 31), less thoroughly evaluated are alterations to vascular mechanics and almost completely unknown are the distal impact of these conditions on muscle vascularity. The results of the present study revealed several key observations. First, while both FH and FCH in mice resulted in an extensive remodeling of the skeletal

muscle microcirculation, the specifics of the remodeling varied between strains. In FH, this remodeling was predominantly at the level of the resistance arterial wall through in increased wall stiffness, while in FCH, the remodeling was localized more at the level of the microvascular networks through an increased rarefaction. Further, the results from the present study identify potential contributors for future interrogation under each condition. Specifically, in FH, predictors such as cholesterol severity and the expression of inflammatory markers for cell attraction/adhesion are implicated for the developing stiffness of the resistance arterial wall. Alternatively, in FCH, the severity of hypertriglyceridemia, the presence of pro-apoptotic markers of inflammation, and alterations to endothelial metabolism of arachidonic acid were identified as correlates of the microvascular network remodeling.

The initial observation from the present study was that, while both familial hypercholesterolemia and familial combined hyperlipidemia can impact vascular wall mechanics, the alterations to wall mechanics with FH (in LDLR mice) develop more rapidly and are more severe than those determined under conditions of FCH (in ApoE mice). While the blunted distention of the arterial wall with increased pressure was in keeping with the observations of a decreased deformability of the vessel wall with chronic dyslipidemia in both strains, this was considerably pronounced in LDLR. When combined with the trend toward an increased wall thickness determined in arteries from LDLR mice, this resulted in significant elevations in wall:lumen ratio, an observation that has also been demonstrated in human subjects with chronic hypercholesterolemia (12, 21) and in the ApoE/LDLR double knockout mouse (6). Taken together, these factors also resulted in the reduction in arterial wall incremental distensibility that was determined for both dyslipidemic strains, although more pronounced in LDLR than in ApoE.

These data describing the mechanical characteristics of the resistance arterial wall become more informative when placed in the context of circumferential stress versus strain relations (Figure 3A) and the relationship between tangential elastic modulus (E_T) versus circumferential stress (Figure 3B). The left-shifting of the stress versus strain relation, as indicated by the increased magnitude of the β coefficient from the exponential equation fit to the data, demonstrates that the stiffness of the resistance artery is increased with FH in LDLR mice. While this process also develops in ApoE, it is considerably less robust and slower to develop. The shape of the E_T versus stress curves reveals an interesting insight into the behavior of the vascular wall. Had these data been best approximated by a

linear model, this would have suggested that the increased stiffness of the arterial wall in LDLR as compared to C57 developed via contributions throughout the range of the intralumenal pressure range. However, these data were better approximated by a non-linear equation (with a superior adjusted r-squared), with the majority of the differences between responses in LDLR and C57 being accounted for over the range of relatively low stresses. From the perspective of functional restraints on perfusion, this suggests that the remodeling that develops in the arterial wall of LDLR may be primarily relevant in terms of restricting diameter at lower intralumenal pressures. Future investigation into the physical alterations to the architecture of the vessel wall, and how this contributes to the shift in these mechanical responses, especially in LDLR mice appears to be well justified.

The other major observation from the present study was that the remodeling of skeletal muscle microvascular networks (microvessel rarefaction) was most pronounced under conditions of familial combined hyperlipidemia. Notably, while the reduction in microvessel density with familial hypercholesterolemia in LDLR mice was present by 22-23 weeks of age, the rarefaction that developed in skeletal muscle of ApoE mice was much more robust, and was clearly identifiable by 12-13 weeks with increasing severity in the older age range. This observation, when taken in context with the impacts of the two models of genetic dyslipidemia on vascular wall mechanics, has a potentially significant implication. Specifically, familial hypercholesterolemia and familial combined hyperlipidemia, while ultimately leading to directionally consistent outcomes in terms of vascular remodeling, preferentially impact different sites along the vascular tree. Familial hypercholesterolemia is associated with an outcome that more rapidly and severely impacts vascular wall distensibility with effects on microvessel density that are delayed and more muted. Conversely, the vascular remodeling associated with familial combined hyperlipidemia is more targeted to microvessel rarefaction, with vascular wall remodeling being less pronounced.

At the outset, the obvious issue to be addressed is how familial hypercholesterolemia and familial combined hyperlipidemia differ with respect to systemic markers of cardiovascular disease risk and vascular function. While both ApoE and LDLR exhibit a significant hypercholesterolemia (Figure 1), this is considerably greater in LDLR than in ApoE. Further, ApoE mice also demonstrate a significant hypertriglyceridemia that is not present in LDLR. The data presented in Figure 5 may provide additional insight in how systemic markers of inflammation may help to

discriminate between the impact of FH and FCH. Plasma levels of TNF-α were much higher in ApoE mice at both ages as compared to LDLR, while levels of MCP-1 exhibited a reversed relationship. However, neither the systemic marker of chronic oxidant stress (nitrotyrosine), nor the inflammatory marker IL-1β, while elevated in ApoE and LDLR versus C57, were able to provide for discrimination between the two dyslipidemic strains.

Previous studies have suggested that alterations to endothelial function can play a critical role in both vascular wall (17, 38) and vascular network (26) remodeling. As such, we utilized three indices of endothelial function in arteries as potential discriminating factors between FH and FCH in terms of vascular remodeling; methacholine-induced nitric oxide bioavailability, and arachidonic acid-induced PGI_2 or TxA_2 production (Figure 6). Vascular nitric oxide bioavailability represented a poor discriminator between ApoE and LDLR mice, as methacholine-induced NO production was rapidly and severely attenuated in both strains. Similarly, the generation of PGI_2 (through the measurement of 6-keto-$PGF_{1\alpha}$) was also a poor discriminator between the dyslipidemic mice, as this parameter was largely intact in both strains at both age ranges. The vascular production of TxA_2, as estimated from the arachidonic acid-induced generation of 11-dehydro-TxB_2 was a stronger discriminator, showing a rapid and significant elevation in ApoE mice as compared to C57, which was maintained to 22-23 weeks. In contrast, 11-dehydro-TxB_2 production in arteries of LDLR was less robust and lagged behind that in arteries in ApoE.

Taken together, these results may begin to provide for a framework distinguishing vascular structural outcomes between familial hypercholesterolemia in LDLR mice and familial combined hyperlipidemia in ApoE mice. In LDLR mice, the development of FH is associated with a more rapid and robust remodeling of the arterial wall, leading to an increased stiffness at lower intralumenal pressures which continues to impact vessel dimension at higher levels of distending pressure. In contrast, microvascular rarefaction develops more slowly under these systemic conditions, reaching significance by 22-23 weeks. Based on the results of the present study, this vascular structural outcome may be well predicted by severity of the hypercholesterolemia and increased MCP-1 expression. This potential linkage has been tentatively identified previously, as in mouse (28, 42) and rabbit (30) models of hypercholesterolemia and in humans afflicted with FH (5, 29), a particularly strong correlation between MCP-1,

cholesterol and atherogenesis has been identified. One of the most compelling possibilities for the role of MCP-1 in contributing to vascular wall remodeling in dyslipidemia was indentified in a recent study by Jagavelu *et al.* (22). In this study, the authors investigated the importance of MAP kinase-activated protein kinase-2 (MK2) in contributing to endothelial dysfunction and atherogenesis. As a key regulator of inflammatory processes, the systemic level of activation of MK2 was correlated with the level of endothelial dysfunction and lipid/macrophage in the vessel wall in LDLR mice, an observation that was independent from the level of pro-atherogenic lipoproteins. With direct relevance to the present study, systemic gene deletion of MK2 in LDLR mice or siRNA silencing of MK2 in endothelial cells was associated with a decreased aortic expression of VCAM-1 and MCP-1, key mediators of macrophage recruitment into the vessel wall. However, to our knowledge, this previous work has not been extended significantly at present and does not include vascular wall mechanics.

In the ApoE mouse, the development of FCH is associated with a microvascular rarefaction that develops quickly, while arterial wall remodeling is delayed. This outcome appears to be well predicted by a severe hypertriglyceridemia in the face of significant hypercholesterolemia, and is well tracked by an increase in the plasma level of TNF-α and the vascular production of TxA_2. While existing studies have linked plasma levels of TNF-α (33) and altered arachidonic acid metabolism (4) with microvascular rarefaction in other models of CVD risk factors, this possibility represents a novel concept in mouse models of genetic dyslipidemia and will require further verification and, assuming validation, investment of resources to determine underlying mechanistic contributors and ameliorative interventions.

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Table 1. Initial values for the dimensions of isolated skeletal muscle resistance arteries from C57, ApoE and LDLR mice at the lowest intralumenal pressure (5 mmHg). * $p<0.05$ vs. 12-13W in that strain ; † $p<0.05$ vs. C57 at that age; ‡ $p<0.05$ vs. ApoE at that age.

	C57		**ApoE**		**LDLR**	
	12-13W	**22-23W**	**12-13W**	**22-23W**	**12-13W**	**22-23W**
Inner Diameter (μm)	45.0±4.5	54.5±5.1	52.7±4.9	58.8±4.5	56.0±4.6	63.8±4.9
Outer Diameter (μm)	93.3±5.0	104.1±4.9	100.7±5.5	108.7±4.9	105.1±4.3	101.5±5.3
Wall Thickness (μm)	48.3±4.0	49.5±3.9	48.0±4.1	49.8±3.9	49.0±4.0	38.0±4.0†‡
CSWA (μm^2)	5250±306	6173±321*	5785±319	6559±266*	6200±228†	4914±285*†‡
Wall:Lumen	1.08±0.10	0.96±0.09	0.96±0.11	0.88±0.09	0.89±0.09	0.60±0.10*†‡

FIGURE LEGENDS

Figure 1. Plasma total cholesterol (Panel A) and triglycerides (Panel B) in C57, ApoE and LDLR mice at 12-13 weeks or 22-23 weeks of age. Data are presented as mean±SEM, n=8 animals for each group. * $p<0.05$ vs. C57 at that age; † $p<0.05$ vs. ApoE at that age.

Figure 2. Passive mechanical characteristics of the skeletal muscle resistance arterial wall in C57, ApoE and LDLR mice at 12-13 weeks or 22-23 weeks of age. Data (mean±SEM) are presented for arterial inner diameter (Panel A), arterial outer diameter (Panel B), arterial wall thickness (Panel C), arterial cross sectional wall area (Panel D), wall:lumen ratio (Panel E) and incremental distensibility (Panel D) over a range of increasing intralumenal pressure, n=8 animals for each group. * $p<0.05$ vs. that strain at 12-13 weeks; † $p<0.05$ vs. C57 at that age; ‡ $p<0.05$ vs. ApoE at that age.

Figure 3. Circumferential wall stress versus strain (Panel A) and tangential elastic modulus versus wall stress (Panel B) relationships in passive skeletal muscle resistance arteries from C57, ApoE and LDLR mice at 12-13 weeks or 22-23 weeks of age. Data are presented as mean±SEM, n=8 animals for each group. * $p<0.05$ vs. that strain at 12-13 weeks; † $p<0.05$ vs. C57 at that age; ‡ $p<0.05$ vs. ApoE at that age.

Figure 4. Gastrocnemius muscle microvessel density in C57, ApoE and LDLR mice at 12-13 weeks or 22-23 weeks of age. Data are presented as mean±SEM, n=8 animals for each group. * $p<0.05$ vs. C57 at that age; † $p<0.05$ vs. ApoE at that age.

Figure 5. Plasma nitrotyrosine (Panel A), TNF-α (Panel B), IL-1β (Panel C) and MCP-1 (Panel D) in C57, ApoE and LDLR mice at 12-13 weeks or 22-23 weeks of age. Data are presented as mean±SEM, n=8 animals for each group. * $p<0.05$ vs. C57 at that age; † $p<0.05$ vs. ApoE at that age.

Figure 6. Data describing the production of nitric oxide (from aortae, Panel A), 6-keto-$PGF_{1\alpha}$ (from pooled arteries, Panel B) or 11-dehydro-TxB_2 (from pooled arteries, Panel C) of C57, ApoE and LDLR. Data, presented as mean±SEM, are shown in response to application of 10^{-4} M methacholine (Panel A) or 10^{-6} M arachidonic acid (Panels B and C). n=6 animals for each group, with each n representing either an aorta or pooled arteries from an individual mouse; please see text for details. * $p<0.05$ vs. C57 at that age; † $p<0.05$ vs. ApoE at that age.

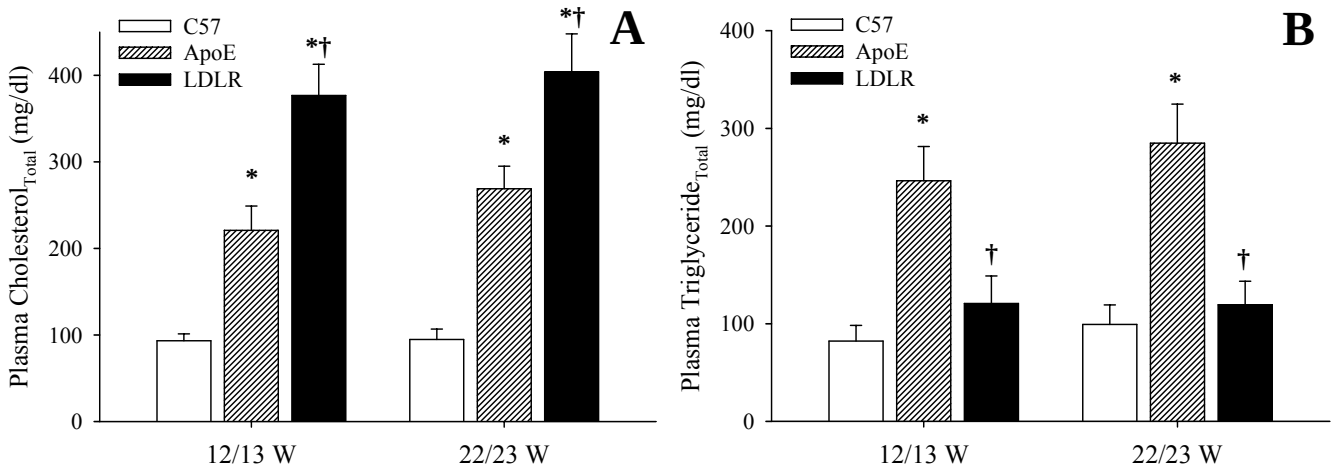
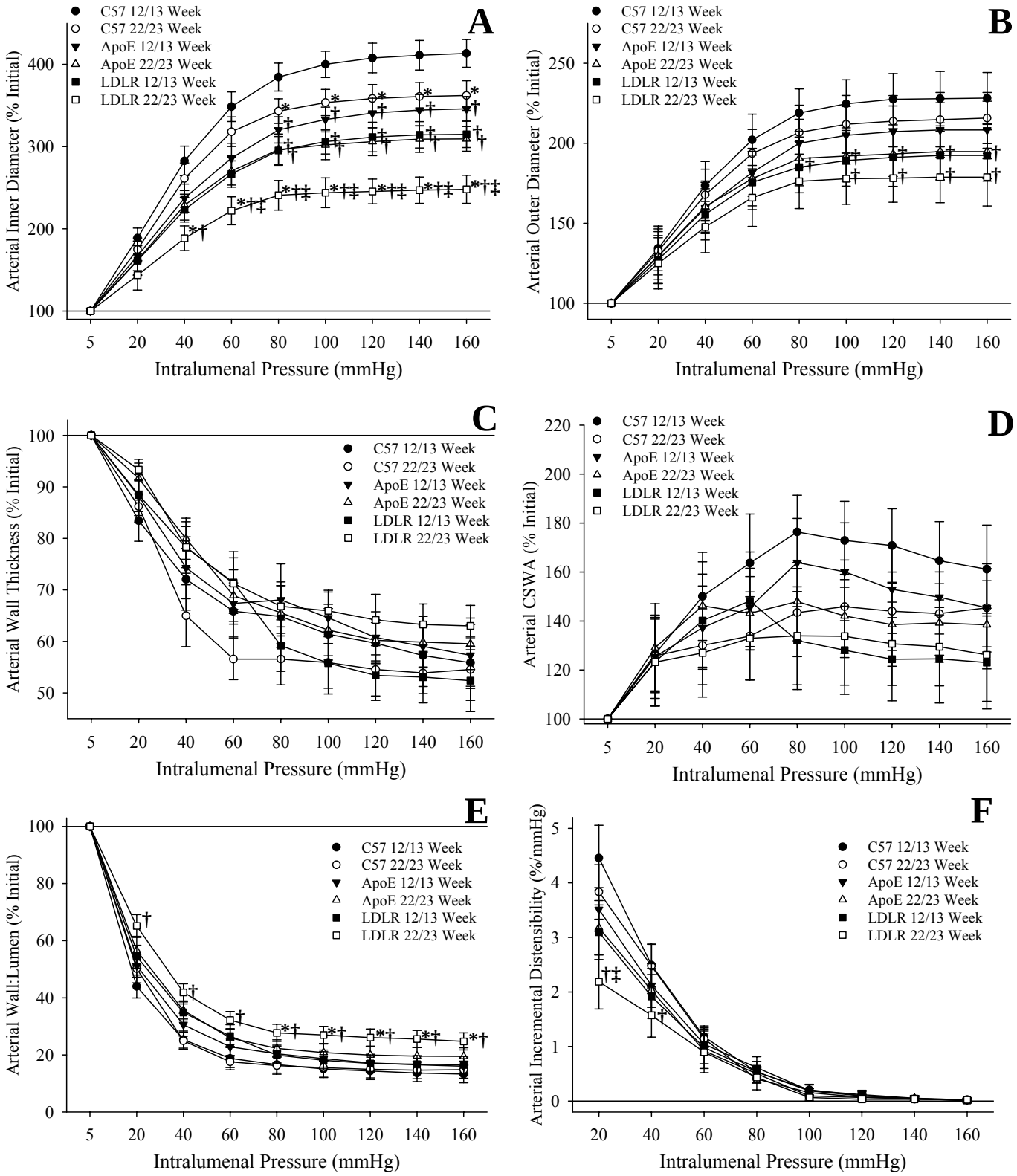


Figure 1.
Stapleton et al.



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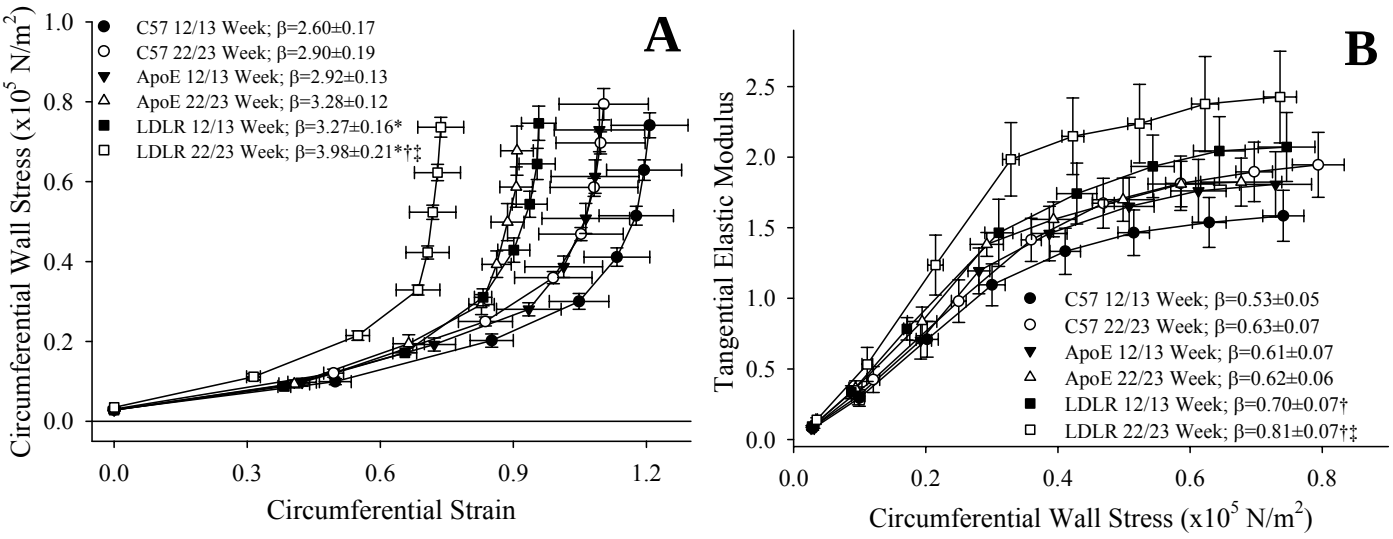

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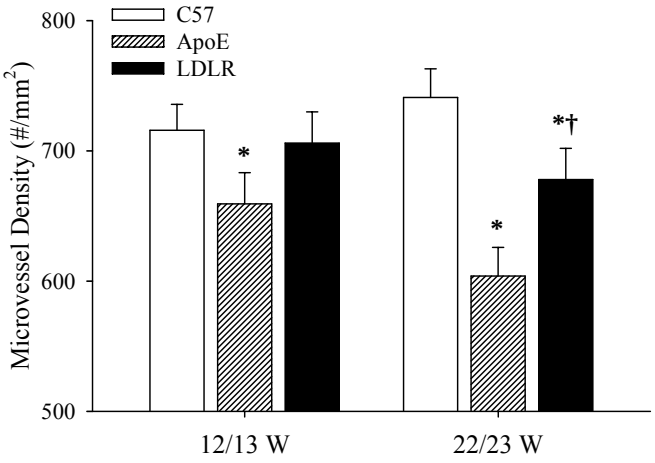

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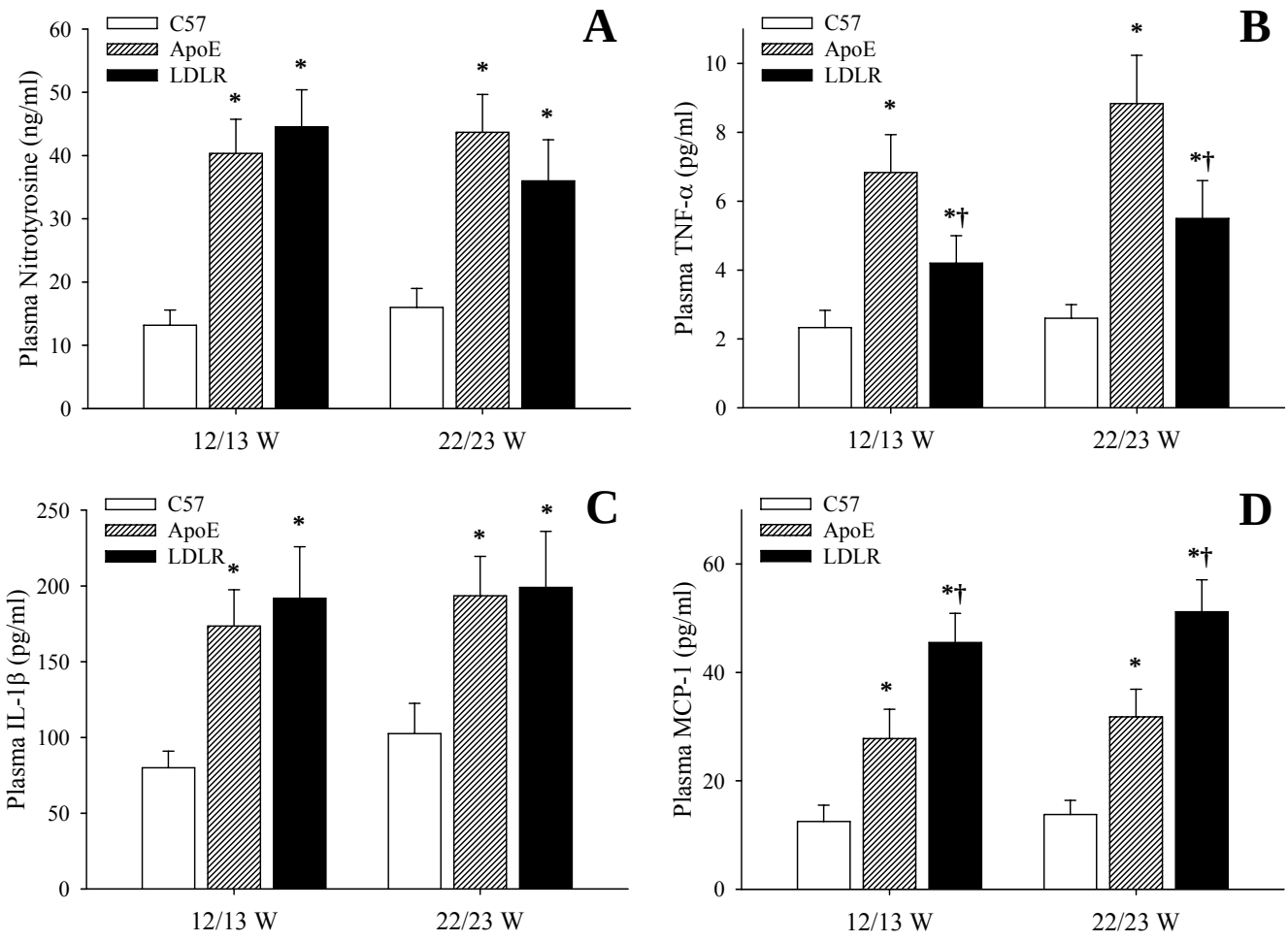

Figure 5.
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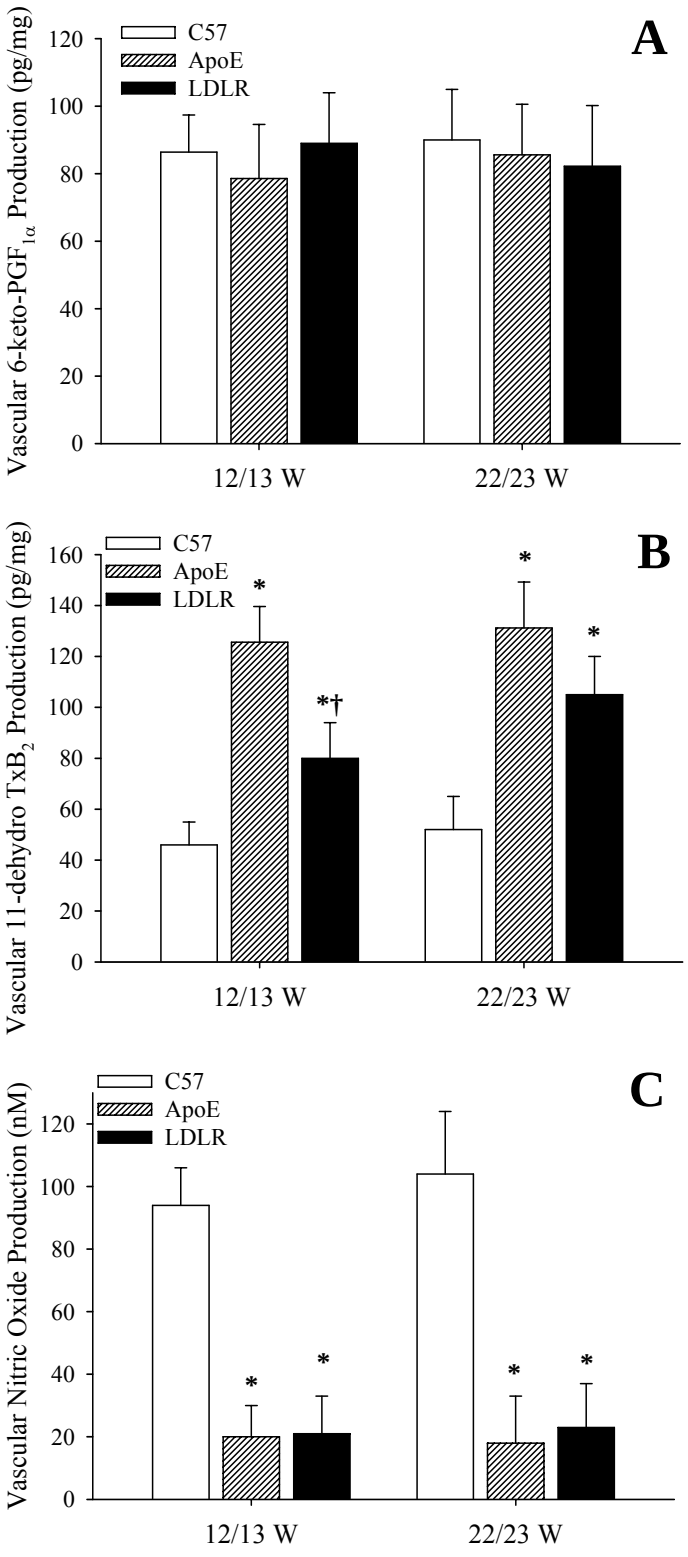

Figure 6.
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ALTERED MECHANISMS OF ENDOTHELIUM-DEPENDENT DILATION IN SKELETAL MUSCLE ARTERIOLES WITH GENETIC HYPERCHOLESTEROLEMIA

Phoebe A. Stapleton[1,3], Adam. G. Goodwill[2,3], Milinda E. James[2,3] and Jefferson C. Frisbee[2,3]

Division of Exercise Physiology[1], Department of Physiology and Pharmacology[2] and
Center for Interdisciplinary Research in Cardiovascular Sciences[3]
West Virginia University School of Medicine, Morgantown, WV 26506

ABSTRACT

With most cardiovascular disease risk factors, endothelium-dependent dilation of skeletal muscle resistance arterioles is compromised, although with hypercholesterolemia, impairments to reactivity are not consistently observed. Using apolipoprotein E (ApoE) and low density lipoprotein receptor (LDLR) gene deletion male mouse models of hypercholesterolemia at 20 weeks of age, the hypothesis tested in this study was that arteriolar dilation would be maintained due to an increased stimulus-induced production of dilator metabolites via cyclooxygenase and cytochrome P450 epoxygenase pathways. Arterioles from both strains demonstrated mild reductions in dilation to hypoxia and acetylcholine versus responses in C57/Bl/6J (C57) controls. However, while inhibition of nitric oxide synthase (NOS) attenuated dilation in arterioles from C57, this effect was absent in ApoE or LDLR. In contrast, cyclooxygenase-dependent portions of dilator reactivity were maintained across the three strains. Notably, while combined NOS and cyclooxygenase inhibition abolished arteriolar responses to hypoxia and acetylcholine in C57, significant reactivity remained in ApoE and LDLR. While inhibition of cytochrome P450 ω-hydroxylase and epoxygenases had no effect on this residual reactivity in ApoE and LDLR, inhibition of 12/15 lipoxygenase with nordihydroguaiaretic acid abolished the residual reactivity. With both hypoxic and methacholine challenges, arteries from ApoE and LDLR demonstrated an increased production of both 12(S)- and 15(S)-HETE, end products of arachidonic acid metabolism via 12/15 lipoxygenase, a response that was not present in C57. These results suggest that with development of hypercholesterolemia, mechanisms contributing to dilator reactivity in skeletal muscle arterioles are modified such that net reactivity to endothelium-dependent stimuli is largely intact.

Key Words: skeletal muscle microcirculation, endothelium-dependent dilation, vascular reactivity, mouse models of cardiovascular disease

INTRODUCTION

It has been well established that development of the hypercholesterolemic condition is a profound risk factor for the evolution of coronary and peripheral arterial disease (1). From an epidemiological perspective, recent studies from the Centers for Disease Control have indicated that under conditions of dyslipidemia, a 10% reduction in total cholesterol levels can result in an estimated 30% reduction in the incidence of coronary artery disease (1). However, while hypercholesterolemia is a clear and profound risk factor for the initiation and progression of peripheral arterial disease, most notably through an increased likelihood for the risk of developing atherosclerotic depositions (16, 44), an understanding of the impact of hypercholesterolemia on the patterns of vascular reactivity has thus far demonstrated considerably less consensus.

In previous studies of human subjects afflicted with genetic dyslipidemia, particularly familial hypercholesterolemia (a genetic disorder resulting in exceptionally high low density lipoprotein [LDL] level, in the face of an otherwise relatively normal lipid profile), arterial dilator reactivity during recovery from brief occlusion (i.e., flow-mediated dilation or reactive hyperemia) was found to be significantly attenuated as compared to responses in normocholesterolemic subjects (2, 6, 8, 20, 29, 39). Further, given the strong dependence of the flow-mediated response on endothelial nitric oxide bioavailability (22), many of these previous results have implicated oxidant radical scavenging of nitric oxide (NO), thus reducing its bioavailability, as an underlying mechanism contributing to impaired reactivity (6, 8, 29, 42). However, results from other studies suggest that, while dilator responses to NO-dependent stimuli are impaired with profound hypercholesterolemia, vasodilation in response to metabolic stimuli are largely intact (7) and may suggest that compensatory mechanisms could be emerging in order to ameliorate the effects of any loss in reactivity owing to an impaired NO bioavailability. Notably, Paniagua *et al.* (34) demonstrated that shear stress-induced dilation of adipose tissue microvessels from hypercholesterolemic subjects was preserved despite a diminished activity of endothelial nitric oxide synthase. The maintenance of vascular reactivity under conditions of hypercholesterolemia has also been suggested by work from Laughlin's group, as these investigators have demonstrated that a high fat, high cholesterol diet had only mild-moderate effects on coronary arteriolar (17) or femoral artery (47) dilation mediated through the vascular endothelium.

Previous studies have suggested that dilator responses of thoracic aortic rings (4, 5) and coronary arteries (25) from hyperlipidemic apolipoprotein E gene deletion mice ingesting a normal diet. However, dilator responses in these mice were profoundly impaired following chronic ingestion of high fat/high cholesterol diets. Given the recent study by Wolfle and de Wit (46), wherein the endothelium-dependent dilator and conducted responses following challenge with acetylcholine were intact in the apolipoprotein E and low density lipoprotein receptor gene deletion mouse models of hypercholesterolemia, and the previous work of Pfister *et al.* (36) which suggests that the pathways of arachidonic acid-induced arterial dilation can be radically altered in hypercholesterolemic rabbits, the purpose of the present study was to determine the effects of profound hypercholesterolemia of genetic origin on mechanisms of endothelium-dependent dilator responses of skeletal muscle resistance arterioles. Using both the apolipoprotein E and low density lipoprotein receptor gene deletion mouse models of hypercholesterolemia, the hypothesis tested in this study was that endothelium-dependent dilator reactivity of skeletal muscle arterioles in these animals would be maintained, despite profound hypercholesterolemia and that this would be manifested through an increased stimulus-induced production of dilator metabolites via cyclooxygenase and cytochrome P450 epoxygenase pathways.

MATERIALS AND METHODS

Animals: The present study used three strains of mice, the C57/Bl/6J (C57) as the control strain and the apolipoprotein E gene deletion (B6.129P2-*Apoe*^{tm1Unc}/J; ApoE) and low density lipoprotein receptor gene deletion (B6.129S7-*Ldlr*^{tm1Her}/J; LDLR) mice on the C57/Bl/6J background. All mice were purchased from Jackson Laboratories (Bar Harbor, ME) at 6 weeks of age. The ApoE gene deletion mouse model of hypercholesterolemia manifests a type III hyperlipidemia in which both plasma cholesterol and triglyceride levels are elevated, although the elevations in LDL are not as severe as in the LDLR gene deletion mouse (37). In contrast, the LDLR gene deletion mouse is a model of human familial hypercholesterolemia, manifesting a profound increase in serum LDL levels while ingesting a normal diet (19).

Male mice of each strain were fed standard chow and drinking water *ad libitum* and were housed in an AAALAC-accredited animal care facility at the West Virginia University Health Sciences Center and all protocols

received prior IACUC approval. At 20 weeks of age, after an overnight fast, mice were anesthetized with injections of sodium pentobarbital (50 $mg \bullet kg^{-1}$ i.p.), and received tracheal intubation to facilitate maintenance of a patent airway. In all mice, a carotid artery was cannulated for determination of arterial pressure. Blood aliquots were drawn from the jugular vein cannula for determination of glucose and insulin (Linco) and a lipid profile levels (Waco). The 20 week age was used in order to allow us to investigate alterations to microvascular structure/function in the presence of chronic dyslipidemia. Further, at this age, the degree of the dysfunction was not so severe that it would not be amenable to amelioration via interventional strategies. Thus, the use of this age range allows us to examine both mechanisms underlying dysfunction as well as the efficacy of interventional strategies for improving microvascular outcomes.

Preparation of Isolated Skeletal Muscle Resistance Arterioles: In anesthetized mice, the intramuscular continuation of the right gracilis artery was surgically removed and cannulated, as described previously for rats (14). These first order arterioles were extended to their approximate *in situ* length and were equilibrated at 80% of the animal's mean arterial pressure in order to approximate the *in vivo* intralumenal pressure experienced by the animal (26). Following equilibration, arteriolar reactivity was evaluated in response to: 1) hypoxia; a reduction in superfusate and perfusate PO_2 from ~135 mmHg (21% O_2) to ~40 mmHg (0% O_2), 2) acetylcholine (10^{-10} M – 10^{-6} M; Sigma), 3) sodium nitroprusside (10^{-10} M – 10^{-6} M; Sigma) and 4) prostacyclin (10^{-10} M – 10^{-6} M; Biomol). Following assessments of arteriolar reactivity, the perfusate and superfusate were replaced with Ca^{2+}-free PSS and vessels were treated with 10^{-7} M phenylephrine until all reactivity and tone were abolished. Subsequently, arteriolar intralumenal pressure was altered, in 20 mmHg increments, between 0 mmHg and 140 mmHg and the inner and outer diameter of arterioles was determined at each pressure. These data were used to calculate arteriolar wall mechanics which were used as indicators of structural alterations to individual microvessels (13).

Removal of the vessel endothelium was accomplished by passing several air bubbles through the perfusate line into the isolated arteriole, the efficacy of which was determined from a loss of all dilator reactivity in response to application of 10^{-6} M acetylcholine (14). To assess the contribution of NO production

or the generation of metabolites via cyclooxygenase as mediators of arteriolar dilator reactivity, isolated vessels were treated with the NO synthase inhibitor L-N^G-nitroarginine methyl ester (L-NAME; 10^{-4} M; Sigma) or the cyclooxygenase antagonist indomethacin (INDO; 10^{-6} M; Sigma), respectively. Additionally, to determine the contribution of metabolites of arachidonic acid mediated via cytochrome P450 enzymes, vessels were treated with the suicide substrate inhibitor 17-octadecynoic acid (17-ODYA; 10^{-5} M; Sigma). Previous studies have demonstrated that 17-ODYA profoundly attenuates both the ω-hydroxylation (producing 20-hydroxyeicosatetraenoic acid; 20-HETE) and epoxygenation (producing epoxyeicosatrienoic acids; EETs) reactions of arachidonic acid through cytochrome P450 (45), thus preventing changes to vascular levels of 20-HETE or EETs as contributing mediators to endothelium-dependent dilation (10, 14, 15, 33). To assess the contribution of lipoxygenase metabolites to the patterns of arteriolar dilation, vessels were treated with nordihydroguaiaretic acid (NDGA; 3×10^{-5} M; Biomol), a selective inhibitor of 12- and 15-lipoxygenases (11, 38, 48).

Measurement of Vascular Nitric Oxide Bioavailability: From each animal, the aorta was removed and vascular NO production was assessed using amperometric sensors (World Precision Instruments). Briefly, aortae were isolated, sectioned longitudinally, pinned in a silastic coated dish and superfused with warmed (37°C) PSS equilibrated with 95% O_2/5% CO_2. The NO sensor (ISO-NOPF 100) was placed in close apposition to the endothelial surface and a baseline level of current was obtained. Subsequently, aortae were exposed to either acute reductions in PO_2 from ~135 mmHg to ~40 mmHg (as described above) or increasing concentrations of methacholine (10^{-10} M –10^{-6} M) and changes in current were determined. To verify that recorded data represented endothelium-dependent NO release, responses were re-evaluated following acute treatment of aortae with L-NAME (10^{-4} M).

Determination of Vascular Metabolites of Arachidonic Acid: Vascular production of 6-keto-prostaglandin $F_{1\alpha}$ (6-keto-$PGF_{1\alpha}$; the breakdown product of PGI_2; ref. 31), 12(S)-hydroxyeicosatetraenoic acid (12(S)-HETE; the stable product from the reduction of 12(S)-hydroperoxytetraenoiceicosatetrenoic acid [12(S)-HpETE], the 12-lipoxyenase metabolite of arachidonic acid; refs. 3, 40, 49) and 15-hydroxyeicosatetrenoic acid (15(S)-HETE; the major

hydroxyl derivative of arachidonic acid when metabolized by 15-lipoxygenase; refs. 3, 27, 43, 48, 49) in response to hypoxia or methacholine within the three mouse groups was assessed using pooled conduit arteries (e.g., femoral, saphenous, iliac, carotid arteries) from each mouse. Vessels were incubated in microcentrifuge tubes in 1 ml of physiological salt solution for 30 minutes under control conditions (21% O_2), after which time either the equilibration gas was switched to 0% O_2 or methacholine (10^{-6} M) was added to the tube for an additional 30 minutes. After the second 30 minute period, the PSS was removed from the tube, frozen in liquid N_2 and stored at -80°C. Metabolite release by the vessels was determined using commercially available EIA kits for 6-keto-$PGF_{1\alpha}$ (Cayman), 12(S)-HETE (Assay Designs) and 15(S)-HETE (Assay Designs).

Data and Statistical Analyses: Active tone of individual arterioles at the equilibration pressure was calculated as $(\Delta D/D_{max})\bullet 100$, where ΔD is the diameter increase from rest in response to Ca^{2+}-free PSS, and D_{max} is the maximum diameter measured at the equilibration pressure in Ca^{2+}-free PSS.

Dilator responses of isolated arterioles following challenge with dilator agonists were fit with the three-parameter logistic equation:

$$y = \min + \left[\frac{\max - \min}{1 + 10^{\log ED_{50} - x}} \right]$$

where y represents the change in arteriolar diameter, "min" and "max" represent the lower and upper bounds, respectively, of the change in arteriolar diameter with increasing agonist concentration, x is the logarithm of the agonist concentration and $\log ED_{50}$ represents the logarithm of the agonist concentration (x) at which the response (y) is halfway between the lower and upper bounds.

The passive arteriolar incremental distensibility (% change in arteriolar diameter/mmHg) was calculated as:

$$DIST_{INC} = \left(\Delta ID \Big/ \left(ID \times \Delta P_{IL} \right) \right) \times 100$$

where ΔID represents the change in internal arteriolar diameter for each incremental change in intralumenal pressure (ΔP_{IL}).

Data describing the vascular production of NO in response to methacholine challenge were fit with a linear regression equation ($y = \alpha_0 + \beta_1(x)$), where (y) represents the nitric oxide production, (x) represents the concentration of methacholine, (α_0) represents an intercept term, and (β_1) represents the rate of change in nitric oxide production for a change in methacholine concentration (slope).

Data are presented as mean±SEM. Statistically significant differences in measured and calculated parameters in the present study were determined using analysis of variance (ANOVA). In all cases, Student-Newman-Keuls post hoc test was used when appropriate and $p<0.05$ was taken to reflect statistical significance.

RESULTS

Table 1 presents baseline characteristics of the three mouse groups used in the present study. While all mice were of comparable mass at 20 weeks of age, LDLR mice demonstrated a statistically significant elevation in mean arterial pressure and insulin resistance as compared to values in either C57 or ApoE. While both ApoE and LDLR manifested a profound hypercholesterolemia, most severe in LDLR animals, these animals also exhibited hypertriglyceridemia as well, which was most substantial in ApoE mice. Additionally, isolated arterioles from ApoE and LDLR mice, while exhibiting comparable diameters with C57 under active conditions, demonstrated a progressive reduction in inner diameter under passive (calcium-free) conditions.

Figure 1 presents dilator reactivity for isolated arterioles from C57, ApoE and LDLR mice in response to challenge with hypoxia (Panel A), and increasing concentrations of acetylcholine (Panel B), sodium nitroprusside (Panel C) and prostacyclin (Panel D). In response to hypoxia or acetylcholine, where arteriolar dilation is strongly endothelium-dependent, responses in vessels from ApoE and LDLR mice were only modestly attenuated as compared to responses determined in C57 control animals. In Panels C (sodium nitroprusside) and D (prostacyclin), where dilator responses to these stimuli are endothelium-independent, arteriolar reactivity was also predominantly intact, demonstrating only mild attenuation.

Data describing the passive mechanical characteristics of the skeletal muscle resistance arteriolar wall in the mouse groups for the present study are summarized in Figure 2. Under Ca^{2+}-free conditions, the increase in vessel

diameter with elevated intralumenal pressure was consistently reduced in isolated arterioles of LDLR as compared to C57, with the majority of this impact being observed at higher levels of pressure (Panel A). While a directionally consistent effect was also determined in a comparison of ApoE to C57, this effect was not as pronounced. However, calculated incremental distensibility, although demonstrating a consistent trend towards a reduced deformability of the vessel wall in the hypercholesterolemic animals, was not significantly different between the three mouse groups (Panel B).

Figure 3 presents vascular reactivity to hypoxia (Panel A) and to increasing concentrations of acetylcholine (Panels B-D) in isolated arterioles of C57, ApoE and LDLR mice following inhibition of nitric oxide synthase, cyclooxygenase and both pathways. In response to hypoxia, C57 mice demonstrated an arteriolar reactivity that was dependent on the release of both NO and PGI_2 from the vascular endothelium. Alternatively, in both ApoE and LDLR mice, arteriolar reactivity to hypoxia demonstrated no statistically significant contribution from nitric oxide synthase, although a significant contribution for metabolites of arachidonic acid via cyclooxygenase remained intact (Panel A). Interestingly, combined inhibition of NOS and cyclooxygenase in both hypercholesterolemic mouse strains did not abolish arteriolar dilation to hypoxia, as a significant reactivity to reduced PO_2 remained intact. Vascular responses to challenge with acetylcholine demonstrated a comparable pattern to that for hypoxia. In arterioles from C57 mice (Panel B), dilation to acetylcholine was an integrated response mediated through contribution of NO and PGI_2. In ApoE (Panel C) and LDLR (Panel D), the NO-dependent portion of arteriolar dilation in response to acetylcholine was attenuated, and the cyclooxygenase-dependent portion remained intact. Further, a significant acetylcholine-induced arteriolar dilation remained in ApoE and LDLR mice despite combined treatment with both L-NAME and indomethacin.

Figure 4 presents data describing vascular NO and PGI_2 production from C57, ApoE and LDLR mice in response to hypoxic and methacholine challenge. Hypoxia-induced NO production was pronounced in aortae from C57 and demonstrated a progressive attenuation in ApoE and LDLR mice, such that this response was entirely abolished in the latter strain (Panel A). A similar pattern was present in response to methacholine challenge, as the agonist-induced generation of NO in C57 was abrogated in both ApoE and LDLR mice (Panel B). In contrast,

production of prostacyclin (from 6-keto-$PGF_{1\alpha}$ levels) from pooled arteries was comparable between C57, ApoE and LDLR in response to either reduced PO_2 (Panel C) or increasing concentrations of methacholine (Panel D).

The role of metabolites of arachidonic acid from cytochrome P450 enzymes in contributing of hypoxia- or acetylcholine-induced vasodilation in C57, ApoE and LDLR mice are summarized in Figure 5. Hypoxic dilation of isolated arterioles from these mice was unaffected by application of 17-ODYA (Panel A), and combined application of 17-ODYA with both L-NAME and indomethacin resulted in a reduction in dilator reactivity that was extremely similar to that determined for NOS and cyclooxygense inhibition alone (as shown in Figure 3, Panel A). This pattern was repeated with acetylcholine challenge in arterioles of C57 (Panel B), ApoE (Panel C) and LDLR (Panel D), as treatment with 17-ODYA alone had minimal impact on acetylcholine-induced dilation and combined treatment with 17-ODYA, L-NAME and indomethacin had an effect that was nearly identical to combined administration of L-NAME and indomethacin in the absence of 17-ODYA.

The effects of 12/15 lipoxygenase inhibition with NDGA on hypoxia- and acetylcholine-induced arteriolar dilation in the mouse groups of the present study are presented in Figure 6. With acute reductions in PO_2 (Panel A), treatment with NDGA alone had no substantial impact on responses in C57 mice, but significantly reduced hypoxic dilation in arterioles of ApoE and LDLR mice. Combined treatment of arterioles with L-NAME, indomethacin and NDGA abolished the responses of vessels from all groups in response to hypoxia. Comparable results were also determined in response to acetylcholine challenge, as NDGA treatment had minimal impact on arteriolar dilation in response to acetylcholine in C57 mice (Panel A), but significantly reduced these responses in ApoE (Panel C) and LDLR (Panel D). Combined treatment with L-NAME, indomethacin, and NDGA abolished all arteriolar acetylcholine-induced reactivity in the hypercholesterolemic mice.

Figure 7 presents data describing the vascular production of 12(S)-HETE and 15(S)-HETE from the mouse groups in the present study in response to hypoxia and challenge with methacholine. The production of 12(S)-HETE from pooled arteries following exposure to hypoxia (Panel A) or methacholine (Panel B) was minimal in C57 mice, but was significantly increased in both ApoE and LDLR mice. Similarly, arterial production of 15(S)-HETE was also significantly increased over that in C57 mice in ApoE and LDLR animals following challenge with

hypoxia (Panel C) or methacholine (Panel D). In all cases, incubation of vessels with NDGA abolished stimulus-induced vascular release of 12(S)- or 15(S)-HETE.

DISCUSSION

While the development of many cardiovascular disease risk factors is associated with profound alterations to vascular reactivity (9, 12, 18) and most commonly with impaired endothelium-dependent dilation (32), the development of hypercholesterolemia has somewhat less predictable effects on vasodilator responses. Impairments to numerous indices of endothelium-dependent dilation have been found in hypercholesterolemic humans (2, 6, 8, 20, 29, 39), but this is not universally observed (7, 34). Additionally, while some animal models of hypercholesterolemia have exhibited blunted patterns of endothelium-dependent dilation (41, 42), studies have also suggested that these impairments can be mild (17, 24, 47), if they are present at all (46). Given this lack of clarity within the existing literature, the present study was designed to evaluate the impact of genetic hypercholesterolemia in mice on endothelium-dependent and independent dilation of skeletal muscle resistance arterioles.

The primary observation of this study was that dilator reactivity of skeletal muscle resistance arterioles of ApoE and LDLR mice was not strikingly different from that determined in C57 control animals. As evident from Figure 1, dilator reactivity to the endothelium-dependent stimuli of hypoxia and acetylcholine, while somewhat blunted, were largely intact in arterioles from both ApoE and LDLR mice. Further, responses to the endothelium-independent stimuli of PGI_2 and the NO donor sodium nitroprusside were also generally intact, and manifested only mild reductions in the magnitude of dilation, if they were present at all. When combined with observations of arteriolar wall mechanics presented in Figure 2, wherein the present results suggest that passive expansion of arteriolar diameter with elevated intralumenal pressure was blunted in ApoE and LDLR mice – resulting in a mild (but not statistically significant) reduction in incremental distensibility – the present results support three initial conclusions: 1) endothelial function, with regard to net dilator reactivity, is largely intact in skeletal muscle resistance arterioles of ApoE and LDLR mice, 2) vascular smooth muscle reactivity to exogenously supplied prostacyclin and nitric oxide appears to be near normal in these microvessels, and 3)

impairments to dilator reactivity at this stage may partially reflect developing alterations to the mechanics of the arteriolar wall rather than simply compromised endothelial or vascular smooth muscle function.

The data presented in Figures 3 and 4 suggest that, while the net dilator reactivity of individual arterioles in response to hypoxia and increasing acetylcholine concentration remains largely intact in ApoE and LDLR in comparison to C57, the predominant signaling molecules which contribute to this reactivity may be substantially altered. Specifically, these results suggest that the portion of hypoxia- or acetylcholine-induced dilation that is mediated by endothelium-dependent generation of NO in C57 is lost with the development of hypercholesterolemia. This interpretation is supported by observations that the impact of L-NAME on dilator reactivity of isolated arterioles in response to hypoxia or challenge with acetylcholine was nearly abolished in ApoE and LDLR, and that the stimulus-induced generation of nitric oxide from either hypoxia or methacholine was profoundly attenuated in arteries of hypercholesterolemic mice. The loss of vascular NO bioavailability with profound hypercholesterolemia has been reported previously (21, 23), and these results support those of previous studies. In contrast, the contribution of endothelium-derived prostacyclin to both hypoxia- and acetylcholine-induced dilation was not substantially impacted by development of the hypercholesterolemic condition in ApoE and LDLR. This was evident in both the consistent impact of indomethacin on dilator responses across the three strains of mice and the comparable level of 6-keto-$PGF_{1\alpha}$ production in arteries of C57, ApoE and LDLR in response to challenge with either hypoxia or methacholine. Notably, the results of these experiments suggest that the arteriolar dilator response to reduced oxygen tension or increased acetylcholine challenge in C57 mice was overwhelmingly the result of the production and release of endothelium-derived nitric oxide and prostacyclin. However, in both ApoE and LDLR models of hypercholesterolemia, significant dilator reactivity to both hypoxia and acetylcholine remained despite pharmacological inhibition of both nitric oxide synthase and cyclooxygenase with L-NAME and indomethacin, respectively. These observations implicate the emergence of an additional dilator signaling pathway which may compensate for the loss of vascular nitric oxide bioavailability during hypercholesterolemia.

Previous studies have suggested that metabolites of arachidonic acid produced via either the ω-hydroxylation (producing 20-HETE) or epoxygenation (producing EETs) reactions of cytochrome P450 enzymes can contribute to arteriolar dilation in response to both hypoxia (14) and acetylcholine (28). However, results from the present study suggest that this is not the case in skeletal muscle resistance arterioles from C57 control mice or with the development of hypercholesterolemia in either ApoE or LDLR mice. Treatment of arterioles with 17-ODYA, either alone or in combination with L-NAME and indomethacin, had no impact on dilator responses to hypoxia or increased concentration of acetylcholine in any of the three mouse strains.

It has previously been demonstrated that the development of diet-induced hypercholesterolemia in rabbits may result in an increased role for lipoxygenase metabolites in contributing to endothelium-dependent dilator reactivity (35). To address the possibility that products of arachidonic acid metabolized through lipoxygenases may contribute to the residual dilation to hypoxia and acetylcholine in ApoE and LDLR following treatment with L-NAME and indomethacin, arterioles were treated with NDGA, either alone or in combination with L-NAME and indomethacin (Figure 6). While NDGA had no significant impact on dilator reactivity in C57, it blunted dilator responses to hypoxia and acetylcholine in arterioles from ApoE and LDLR when given alone, and abolished responses in arterioles from these strains when given following pre-treatment with L-NAME and indomethacin. These results provided compelling evidence that the generation of metabolites of arachidonic acid via lipoxygenase may provide a compensatory mechanism to maintain arteriolar dilator reactivity in ApoE and LDLR. In support of this concept, data presented in Figure 7 provided evidence that vascular production of 12(S)-HETE and 15(S)-HETE, from 12- and 15-lipoxygenase, respectively, although minimal in C57 in response to either hypoxia or methacholine challenge, was profoundly elevated in arteries of both ApoE and LDLR following exposure to both of these stimuli.

Recent studies have provided some compelling insight into the patterns of arteriolar reactivity in mice with diet-induced hypercholesterolemia. In wild type mice, two weeks of high cholesterol diet has previously been demonstrated to result in an impaired dilator reactivity of cremasteric arterioles *(in situ)* to 10^{-5} M acetylcholine (41). However, additional study from Kim *et al.* (24) suggested that the impairments to arteriolar

dilation with diet-induced hypercholesterolemia in wild type mice may be a function of the arteriolar proximity to a paired venule, as the degree of dilator impairment was inversely proportional to diffusion distance from the venule. Interestingly, both of these studies have provided evidence that the dysfunction may be most tightly predicted by profound elevations in oxidant stress (24, 41) and elevations in P-selectin mediated cellular adhesion (24). The results from these previous studies may provide compelling avenues for future study.

Throughout these studies, the production of vasodilator metabolites in response to challenge with either hypoxia or methacholine was determined from larger conduit arteries or aortic segments. These data were then employed to provide insight into the mechanical responses of skeletal muscle resistance arterioles following exposure to either reductions in oxygen tension or challenge with acetylcholine. As a result, these results should be interpreted with some caution, as mechanisms underlying vascular reactivity in response to specific stimuli are not necessarily consistent across all vessels and can demonstrate considerable heterogeneity. Ongoing studies will be needed to determine if the present results acquired using conduit arteries are maintained in the peripheral microcirculation.

Taken together, the results of the present study suggest that with the development of genetic hypercholesterolemia in ApoE and LDLR mice, the dilator reactivity of skeletal muscle resistance arterioles is largely intact, although the signaling mechanisms responsible for these responses are altered. While the contribution of prostacyclin to endothelium-dependent dilation appears to be maintained, vascular levels of nitric oxide bioavailability are dramatically reduced. However, with the development of hypercholesterolemia, the production of dilator metabolites via 12- and 15-lipoxygenase emerges as a compensatory mechanism in ApoE and LDLR, and help to maintain net dilator reactivity in these vessels despite the loss of components dependent on nitric oxide bioavailability. Focused effort into the signaling mechanisms responsible for the reduction in vascular nitric oxide bioavailability and the progressive compensation for this loss by lipoxygenases may represent exciting avenues for future investigation. Additionally, the consequences of both the chronic reductions in nitric oxide bioavailability and the increased generation of arachidonic acid metabolites via lipoxygenases for other microvascular outcomes (e.g., microvessel network structure, wall

mechanics, anti-thrombotic processes, spatio-temporal regulation of perfusion) will also require considerable future study.

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Table 1. Baseline characteristics of mice and individual arterioles used in the present study. * $p<0.05$ vs. C57; † $p<0.05$ vs. ApoE.

	C57	**ApoE**	**LDLR**
Mass (g)	32±1	34±1	31±1
MAP (mmHg)	85±2	89±3	102±4*
$[Glucose]_{blood}$ (mg/dl)	91±5	108±10	117±6*
$[Insulin]_{plasma}$ (ng/ml)	1.2±0.2	1.5±0.8	3.1±0.6*
$[Total\ Cholesterol]_{plasma}$ (mg/dl)	90±10	302±19*	425±29*†
$[LDL\ Cholesterol]_{plasma}$ (mg/dl)	52±6	285±15*	396±24*†
$[Triglycerides]_{plasma}$ (mg/dl)	86±6	188±15*	124±14*†
Inner Diameter – Active (μm)	59±4	54±3	56±4
Inner Diameter – Passive (μm)	155±5	146±5	133±7*
Active Tone (%)	61±4	63±4	58±4

FIGURE LEGENDS

Figure 1. Data describing the dilator reactivity of isolated skeletal muscle resistance arterioles of C57, ApoE and LDLR mice in response to hypoxia (Panel A), and increasing concentrations of acetylcholine (Panel B), sodium nitroprusside (Panel C) and prostacyclin (Panel D). Data, presented as mean±SEM, are shown for arterioles under control conditions and for endothelium-dependent stimuli (hypoxia and acetylcholine), following removal of the vascular endothelium (please see text for details). n=8 animals for each strain; * $p<0.05$ vs. C57; † $p<0.05$ vs. control within that strain.

Figure 2. Data, presented as mean±SEM, describing intralumenal pressure-induced expansion (Panel A) and arteriolar wall incremental distensibility (Panel B) of isolated skeletal muscle microvessels from C57, ApoE and LDLR mice under passive (Ca^{2+}-free) conditions. n=8 animals for each group; * $p<0.05$ vs. C57.

Figure 3. Data describing the dilator responses of isolated skeletal muscle resistance arterioles of C57, ApoE and LDLR mice in response to hypoxia (Panel A) and increasing concentrations of acetylcholine (Panels B-D). Data, presented as mean±SEM, are shown for arterioles under control conditions, and following pharmacological inhibition of nitric oxide synthase with L-NAME, cyclooxygenase with indothemation (INDO) or combined inhibition of both enzymatic pathways (please see text for details). n=5-10 animals for each group; * $p<0.05$ vs. control conditions, † $p<0.05$ vs. no response.

Figure 4. Data describing the production of nitric oxide (from aortae) or 6-keto-$PGF_{1\alpha}$ (from pooled arteries) of C57, ApoE and LDLR in response to hypoxia (Panels A and C, respectively) or increasing concentrations of methacholine (Panels B and D, respectively). Data, presented as mean±SEM, are shown under control conditions, and following pharmacological inhibition of NO synthase with L-NAME or cyclooxygenase with indothemation (INDO), as appropriate. n=8 animals for each group, with each n representing either an aortae or pooled arteries

from an individual mouse; please see text for details. * $p<0.05$ vs. respective control; † $p<0.05$ vs. C57 under that condition; ‡ vs. ApoE under that condition.

Figure 5. Data describing the dilator responses of isolated skeletal muscle resistance arterioles of C57, ApoE and LDLR mice in response to hypoxia (Panel A) and increasing concentrations of acetylcholine (Panels B-D). Data, presented as mean±SEM, are shown for arterioles under control conditions, and following pharmacological inhibition of cytochrome P450 enzymes with 17-ODYA, either alone or in combination with L-NAME and indothemation (INDO). n=6-7 animals for each group; * $p<0.05$ vs. control conditions in that strain; † $p<0.05$ vs. treatment with 17-ODYA alone; ‡ $p<0.05$ vs. no response.

Figure 6. Data, presented as mean±SEM, describing the dilator responses of isolated skeletal muscle resistance arterioles of C57, ApoE and LDLR mice in response to hypoxia (Panel A) and increasing concentrations of acetylcholine (Panels B-D). Data are shown for arterioles under control conditions, and following pharmacological inhibition of lipoxygenase enzymes with NGDA, either alone or in combination with L-NAME and indothemation (INDO). n=6-7 animals for each group; * $p<0.05$ vs. control conditions; † $p<0.05$ vs. treatment with NDGA alone.

Figure 7. Data describing the production of 12(S)-HETE or 15(S)-HETE from pooled arteries of C57, ApoE and LDLR in response to hypoxia (Panels A and C) or increasing concentrations of methacholine (Panels B and D). Data, presented as mean±SEM, are shown for arteries under control conditions, and following pharmacological inhibition of lipoxygenases with NDGA. n=6 animals for each group, with each n representing pooled arteries from an individual mouse; please see text for details. * $p<0.05$ vs. respective control; † $p<0.05$ vs. C57 under that condition.

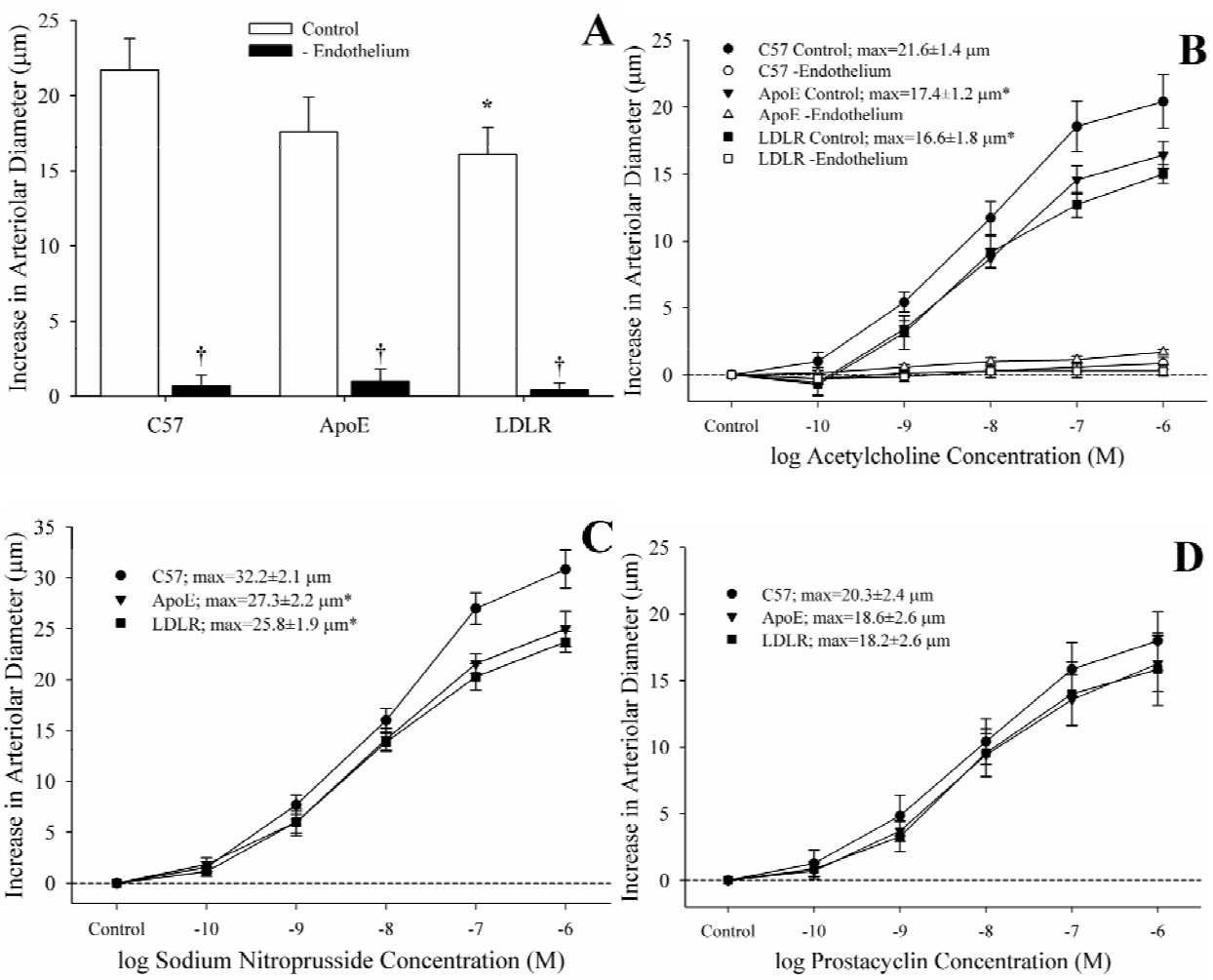
A
Control
- Endothelium
Increase in Arteriolar Diameter (μm)
C57
ApoE
LDLR
B
C57 Control; max=21.6±1.4 μm
C57 -Endothelium
ApoE Control; max=17.4±1.2 μm*
ApoE -Endothelium
LDLR Control; max=16.6±1.8 μm*
LDLR -Endothelium
log Acetylcholine Concentration (M)
C
C57; max=32.2±2.1 μm
ApoE; max=27.3±2.2 μm*
LDLR; max=25.8±1.9 μm*
log Sodium Nitroprusside Concentration (M)
D
C57; max=20.3±2.4 μm
ApoE; max=18.6±2.6 μm
LDLR; max=18.2±2.6 μm
log Prostacyclin Concentration (M)

Figure 1

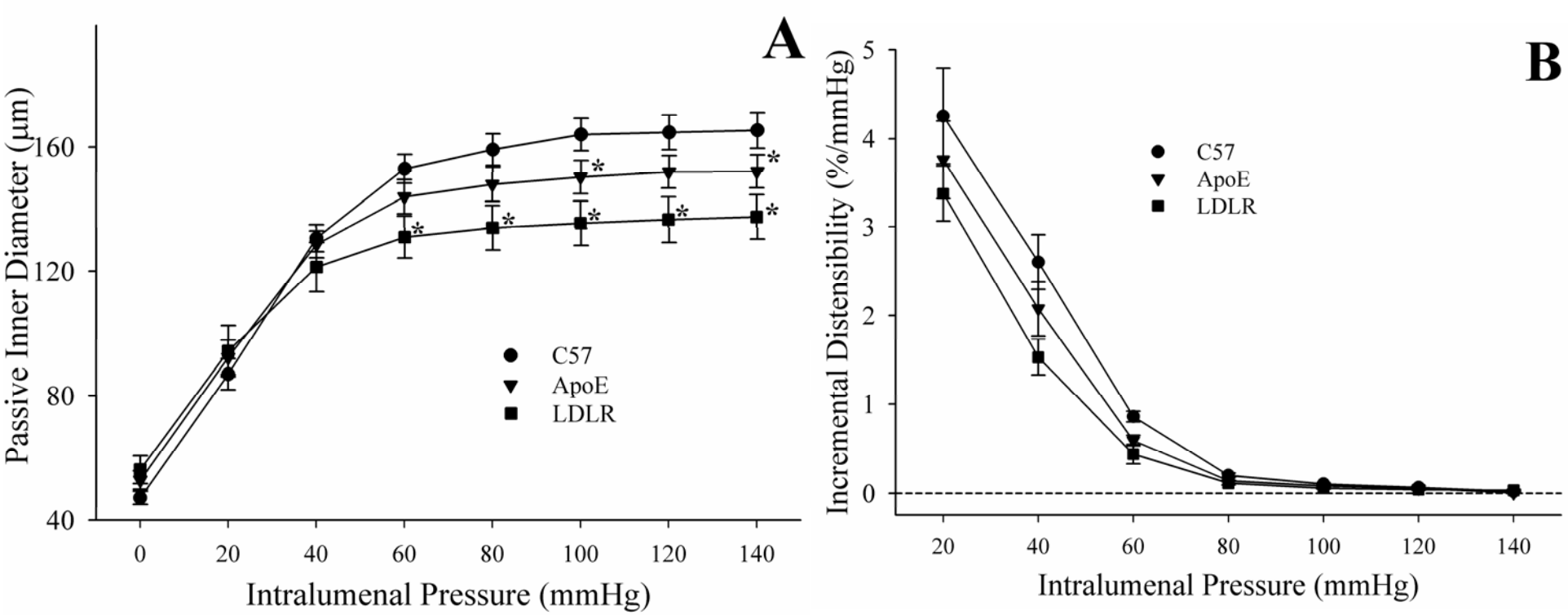
A
Passive Inner Diameter (µm)
160
120
80
40
0
20
40
60
80
100
120
140
Intralumenal Pressure (mmHg)
C57
ApoE
LDLR
*
B
Incremental Distensibility (%/mmHg)
5
4
3
2
1
0
20
40
60
80
100
120
140
Intralumenal Pressure (mmHg)
C57
ApoE
LDLR

Figure 2

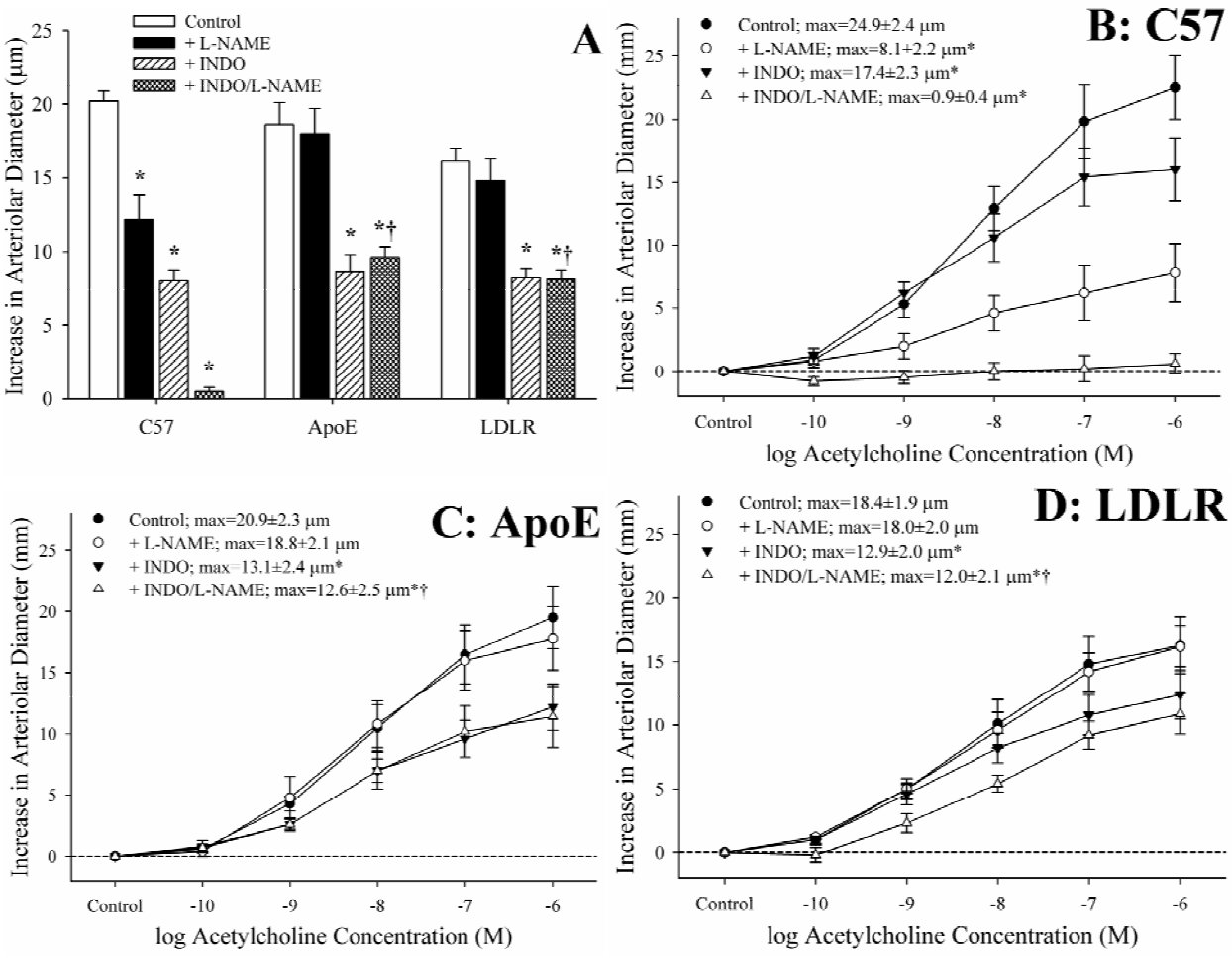
A
Control
+ L-NAME
+ INDO
+ INDO/L-NAME
Increase in Arteriolar Diameter (µm)
C57
ApoE
LDLR
B: C57
Control; max=24.9±2.4 µm
+ L-NAME; max=8.1±2.2 µm*
+ INDO; max=17.4±2.3 µm*
+ INDO/L-NAME; max=0.9±0.4 µm*
Increase in Arteriolar Diameter (mm)
log Acetylcholine Concentration (M)
C: ApoE
Control; max=20.9±2.3 µm
+ L-NAME; max=18.8±2.1 µm
+ INDO; max=13.1±2.4 µm*
+ INDO/L-NAME; max=12.6±2.5 µm*†
D: LDLR
Control; max=18.4±1.9 µm
+ L-NAME; max=18.0±2.0 µm
+ INDO; max=12.9±2.0 µm*
+ INDO/L-NAME; max=12.0±2.1 µm*†

Figure 3

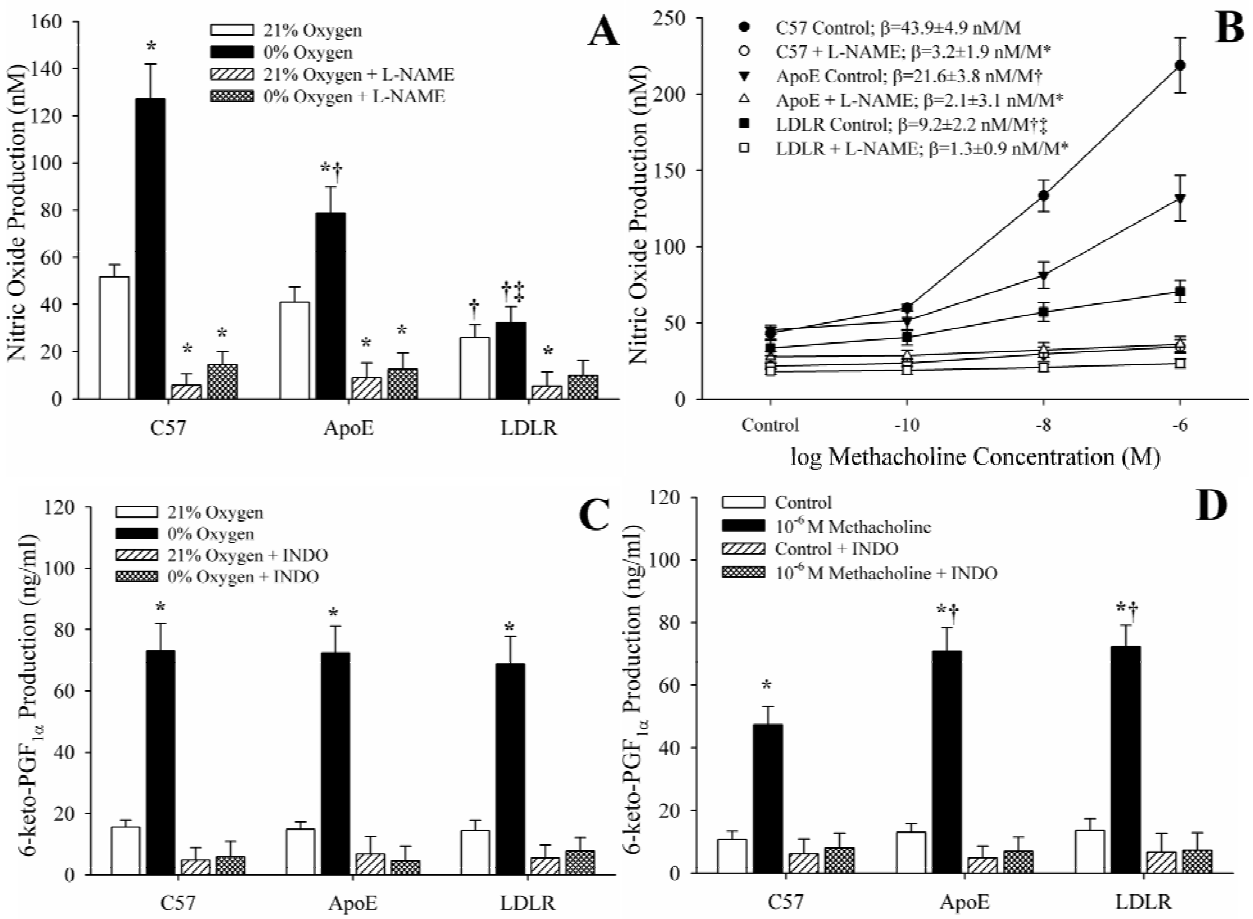
A
21% Oxygen
0% Oxygen
21% Oxygen + L-NAME
0% Oxygen + L-NAME
Nitric Oxide Production (nM)
C57
ApoE
LDLR
B
C57 Control; β=43.9±4.9 nM/M
C57 + L-NAME; β=3.2±1.9 nM/M*
ApoE Control; β=21.6±3.8 nM/M†
ApoE + L-NAME; β=2.1±3.1 nM/M*
LDLR Control; β=9.2±2.2 nM/M†‡
LDLR + L-NAME; β=1.3±0.9 nM/M*
Nitric Oxide Production (nM)
Control
-10
-8
-6
log Methacholine Concentration (M)
C
21% Oxygen
0% Oxygen
21% Oxygen + INDO
0% Oxygen + INDO
6-keto-PGF1α Production (ng/ml)
C57
ApoE
LDLR
D
Control
10-6 M Methacholine
Control + INDO
10-6 M Methacholine + INDO
6-keto-PGF1α Production (ng/ml)
C57
ApoE
LDLR

Figure 4

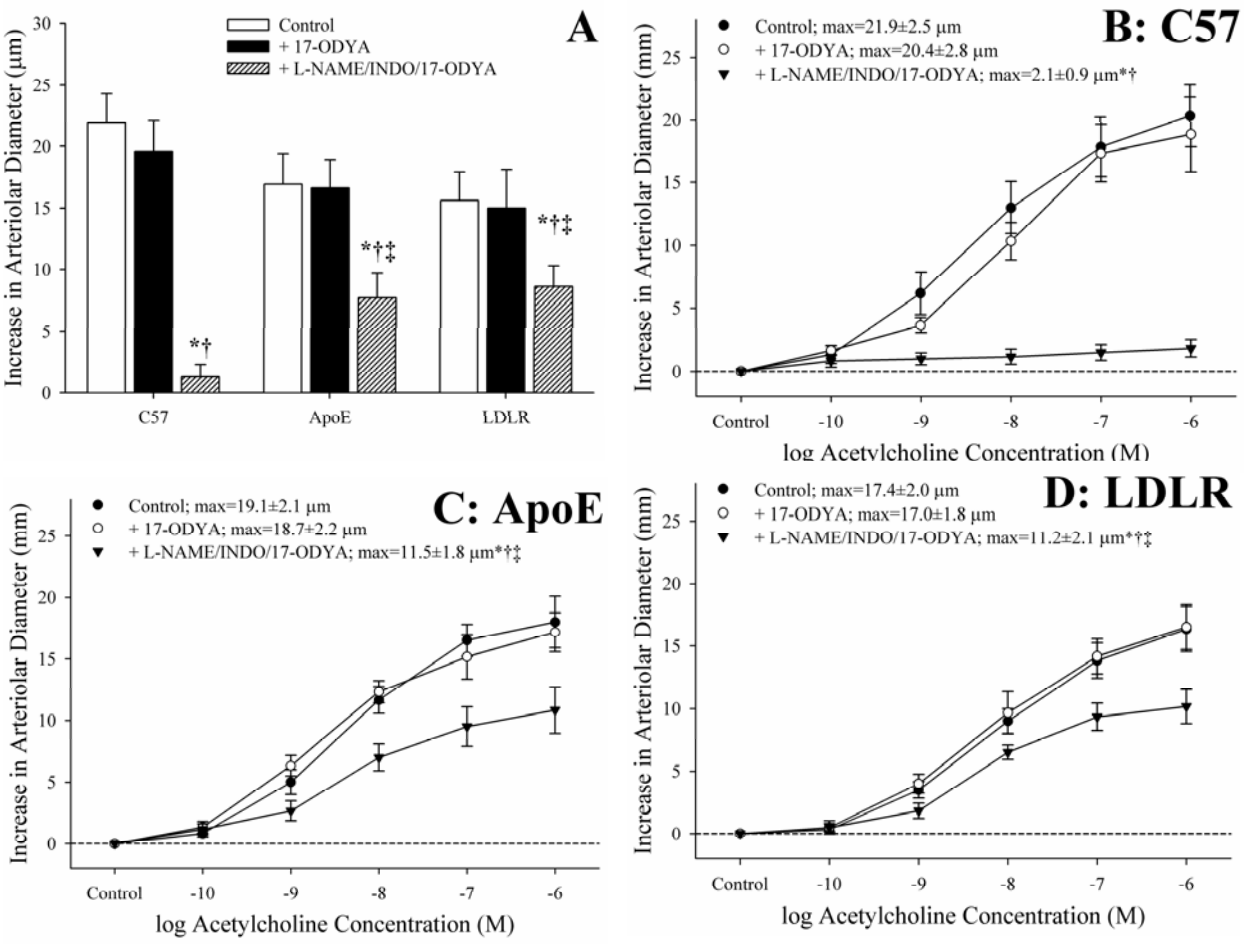
A
Control
+ 17-ODYA
+ L-NAME/INDO/17-ODYA
Increase in Arteriolar Diameter (µm)
C57
ApoE
LDLR
B: C57
Control; max=21.9±2.5 µm
+ 17-ODYA; max=20.4±2.8 µm
+ L-NAME/INDO/17-ODYA; max=2.1±0.9 µm*†
Increase in Arteriolar Diameter (mm)
log Acetylcholine Concentration (M)
C: ApoE
Control; max=19.1±2.1 µm
+ 17-ODYA; max=18.7±2.2 µm
+ L-NAME/INDO/17-ODYA; max=11.5±1.8 µm*†‡
D: LDLR
Control; max=17.4±2.0 µm
+ 17-ODYA; max=17.0±1.8 µm
+ L-NAME/INDO/17-ODYA; max=11.2±2.1 µm*†‡

Figure 5

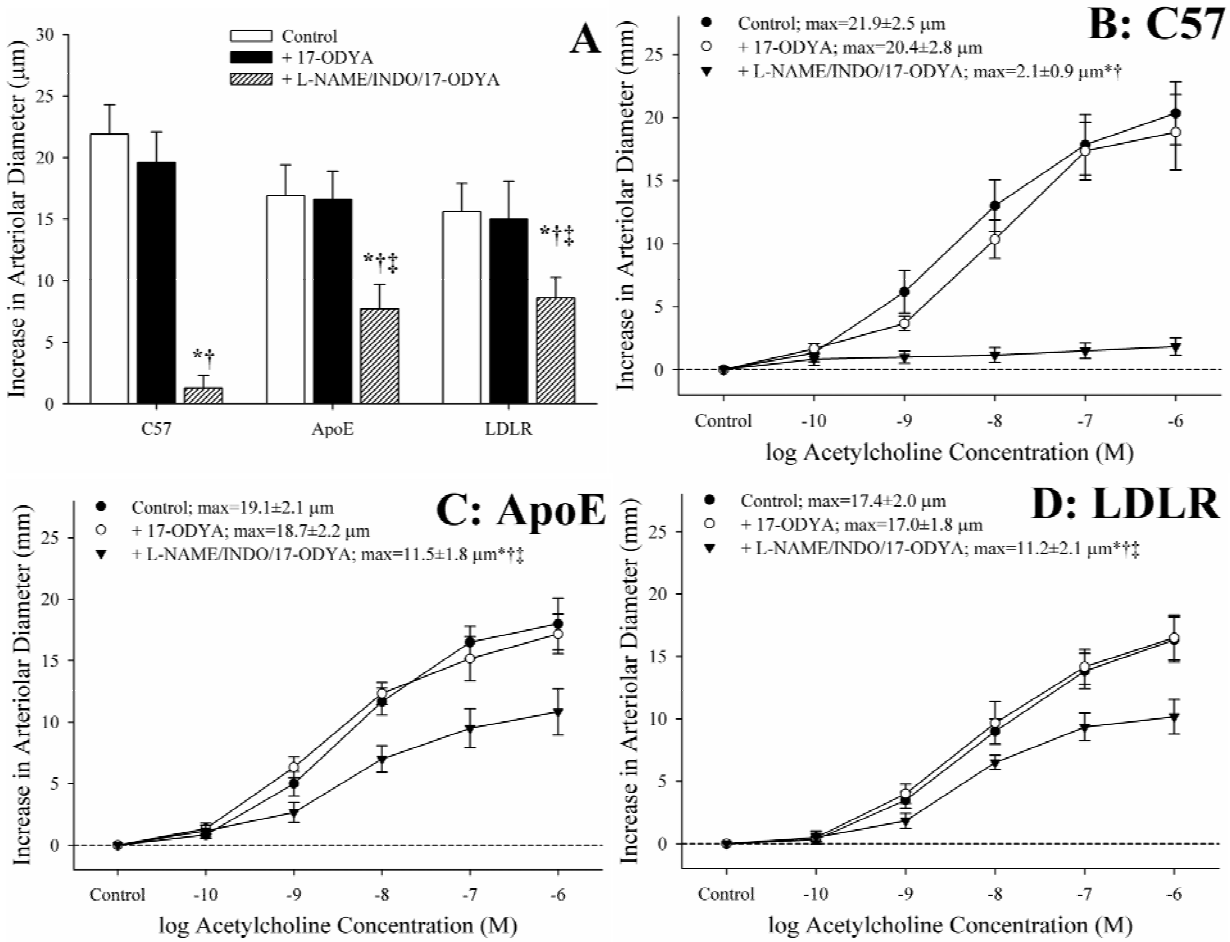
A
Control
+ 17-ODYA
+ L-NAME/INDO/17-ODYA
Increase in Arteriolar Diameter (μm)
C57
ApoE
LDLR
B: C57
Control; max=21.9±2.5 μm
+ 17-ODYA; max=20.4±2.8 μm
+ L-NAME/INDO/17-ODYA; max=2.1±0.9 μm*†
Increase in Arteriolar Diameter (mm)
log Acetylcholine Concentration (M)
C: ApoE
Control; max=19.1±2.1 μm
+ 17-ODYA; max=18.7±2.2 μm
+ L-NAME/INDO/17-ODYA; max=11.5±1.8 μm*†‡
D: LDLR
Control; max=17.4±2.0 μm
+ 17-ODYA; max=17.0±1.8 μm
+ L-NAME/INDO/17-ODYA; max=11.2±2.1 μm*†‡

Figure 5

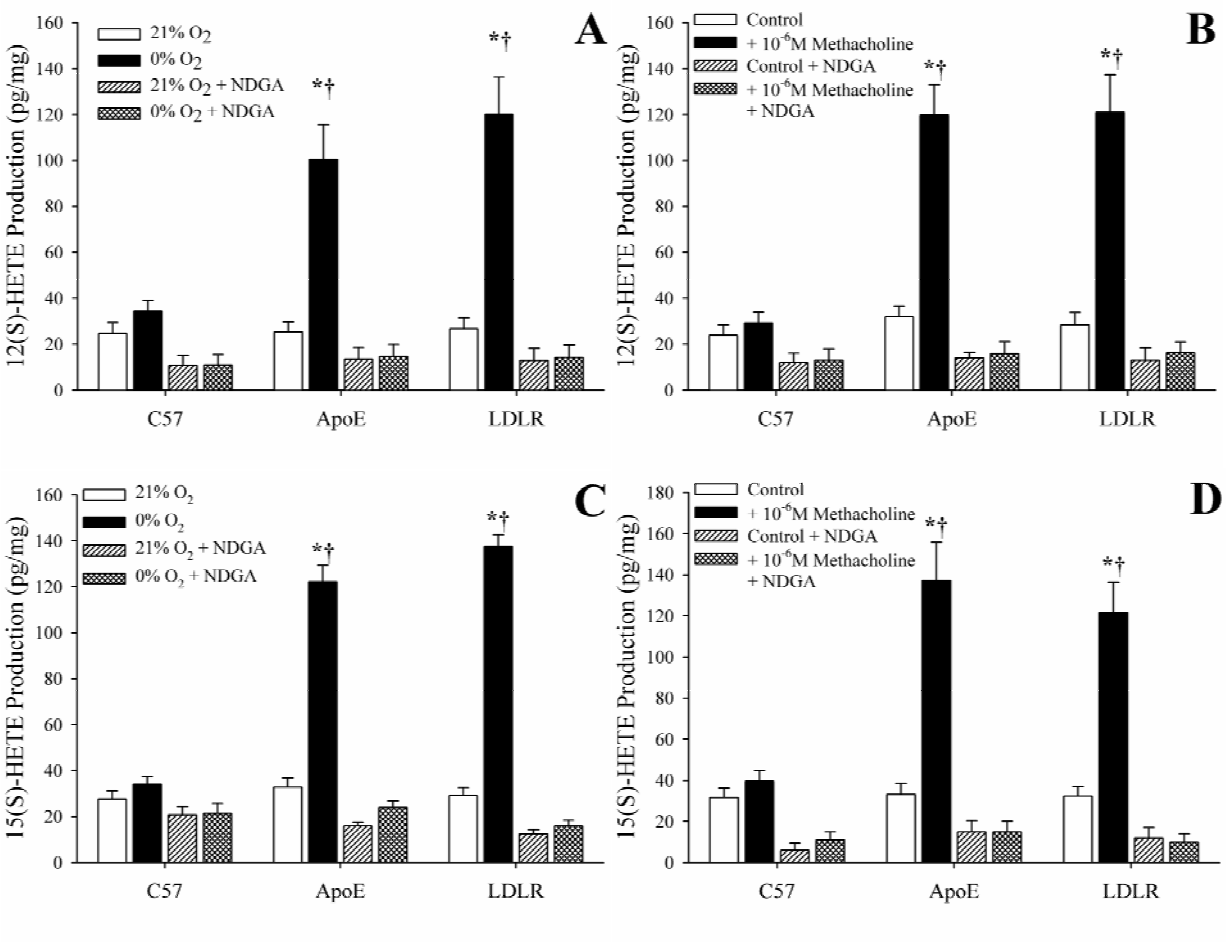
A
B
C
D
12(S)-HETE Production (pg/mg)
15(S)-HETE Production (pg/mg)
21% O2
0% O2
21% O2 + NDGA
0% O2 + NDGA
Control
+ 10-6M Methacholine
Control + NDGA
+ 10-6M Methacholine + NDGA
*†
C57
ApoE
LDLR

Figure 7

INCREASED ARACHIDONIC ACID-INDUCED THROMBOXANE GENERATION IMPAIRS SKELETAL MUSCLE ARTERIOLAR DILATION WITH GENETIC DYSLIPIDEMIA

Adam. G. Goodwill[1,4], Phoebe A. Stapleton[2,4], Milinda E. James[1,4], Alexandre C. d'Audiffret[3,4] and Jefferson C. Frisbee[1,4]

Department of Physiology and Pharmacology[1], Division of Exercise Physiology[2], Division of Vascular and Endovascular Surgery[3] and Center for Interdisciplinary Research in Cardiovascular Sciences[4]
West Virginia University School of Medicine, Morgantown, WV 26506

Running Head: Hypercholesterolemia, thromboxane, and arteriolar reactivity

ABSTRACT

Objective: To determine if arachidonic acid (AA)-induced skeletal muscle arteriolar dilation is altered with hypercholesterolemia in ApoE and LDLR gene deletion mice fed normal diet. This study also determined contributors to altered AA-induced dilation between dyslipidemic mice and controls; C57/Bl/6J (C57).

Methods: Gracilis muscle arterioles were isolated, with mechanical responses assessed following challenge with AA under control conditions and after elements of AA metabolism pathways were inhibited. Conduit arteries from each strain were used to assess AA-induced production of PGI_2 and TxA_2.

Results: Arterioles from ApoE and LDLR exhibited a blunted dilation to AA versus C57. While responses were cyclooxygenase-dependent in all strains, inhibition of thromboxane synthase or blockade of PGH_2/TxA_2 receptors improved dilation in ApoE and LDLR only. AA-induced generation of PGI_2 was comparable across strains, although TxA_2 generation was increased in ApoE and LDLR. Arteriolar reactivity to PGI_2 and TxA_2 was comparable across strains. Treatment with TEMPOL improved dilation and reduced TxA_2 production with AA in ApoE and LDLR.

Conclusions: These results suggest that AA-induced arteriolar dilation is constrained in ApoE and LDLR via an increased production of TxA_2. While partially due to elevated oxidant stress, additional mechanisms contribute which are independent of acute alterations in oxidant tone.

INTRODUCTION

Dyslipidemia, and specifically hypercholesterolemia, has repeatedly been demonstrated to represent a strong predisposing risk factor for the development of coronary and peripheral arterial disease (1). While this increased risk for the progression of vascular disease with hypercholesterolemia is most commonly associated with an increased predisposition for the development of atherothromboses, atherosclerotic lesions and plaque depositions (3, 10, 26), investigations into the impact of hypercholesterolemia on vascular reactivity and endothelial function, potentially as contributing mechanisms to vascular disease, is less clearly understood.

While some disparity in the prevailing literature exists (25), the general consensus is that the development of hypercholesterolemia is usually associated with a significant reduction in the bioavailability of endothelium-derived nitric oxide (5, 6, 23), with the relatively predictable ensuing outcome of an impaired vascular reactivity in response to stimuli that are considered to have a significant contribution from this signaling molecule/pathway (i.e., flow-mediated dilation; ref. 12). In our recent study, we provided evidence suggesting that development of familial hypercholesterolemia (a genetic disorder resulting in exceptionally high low density lipoprotein [LDL] level, in the face of an otherwise relatively normal lipid profile) in the LDL receptor gene deletion mouse or type III hyperlipidemia (a condition wherein both LDL and plasma triglycerides are significantly elevated) in the apolipoprotein E gene deletion mouse, was associated with a near complete abolition of the bioavailability of endothelium-derived nitric oxide in response to imposed stimuli (22). However, this loss of vascular nitric oxide bioavailability did not result in a profound reduction in dilator reactivity, as an increased generation of dilator signaling molecules through 12/15 lipoxygenases emerged with evolution of the dyslipidemia (22), suggesting that alterations to the metabolism of arachidonic acid may be associated with hypercholesterolemia, and that these can have profound consequences for vascular function.

In 1996, the work of Pfister and colleagues (16, 17) strongly suggested that diet-induced hypercholesterolemia in rabbits can lead to changes in arachidonic acid metabolism, mediated via lipoxyegnase are cytochrome P450 epoxygenase enzymes, causing profound alterations to dilator reactivity

determined in isolated aortic segments. Additionally, Srisawat *et al.* (21), while providing additional evidence that diet-induced hypercholesterolemia results in impaired endothelium-dependent dilation in aortic rings, determined that chronic treatment with indomethacin improved endothelial function, and was associated with reductions in urinary levels of 2,3-dinor-thromboxane B_2 and 8-iso-$PGF_{2\alpha}$, a stable urinary breakdown product of thromboxane A_2 and a marker of chronic oxidant stress, respectively. Most recently, Pfister demonstrated that impairments to endothelium-dependent dilation in aortic rings of hypercholesterolemic rabbits were diminished in a subgroup of animals lacking a functional thromboxane receptor (15). These previous results suggest that a contributing mechanism underlying alterations to vascular reactivity under conditions of hypercholesterolemia may involve both elevated vascular oxidant stress and metabolism of arachidonic acid through cyclooxygenase pathways. However, given recent observations in our laboratory (22) and by others (25) suggesting that alterations to endothelium-dependent reactivity may reflect the specific challenge imposed rather than a global impairment, we examined alterations to dilator reactivity in response to direct challenge with arachidonic acid itself, wherein the bioavailability of endothelium-derived nitric oxide is not a significant contributing element to the net mechanical response. Using both apolipoprotein E and LDL receptor gene deletion mouse models of hypercholesterolemia, the hypothesis tested in the present study was that arachidonic acid-induced dilator reactivity of skeletal muscle arterioles would be impaired in the presence of profound dyslipidemia and that this would be the result of alterations to either the production or vascular reactivity to metabolites of arachidonic acid via cyclooxygenase, owing to the presence of an elevated oxidant stress.

MATERIALS AND METHODS

Animals: The present study used three strains of mice, the C57/Bl/6J (C57) as the control strain and the apolipoprotein E gene deletion (B6.129P2-*Apoe*^{tm1Unc}/J; ApoE) and low density lipoprotein receptor gene deletion (B6.129S7-*Ldlr*^{tm1Her}/J; LDLR) mice on the C57/Bl/6J background. All mice were purchased from Jackson Laboratories (Bar Harbor, ME) at 6 weeks of age. The ApoE mouse manifests type III hyperlipidemia, in which both plasma cholesterol and triglyceride levels are elevated, although the

elevations in LDL are not as severe as in the LDLR gene deletion mouse (19). In contrast, the LDLR mouse is a model of human familial hypercholesterolemia, manifesting a profound increase in serum LDL levels while ingesting a normal diet (11).

Male mice of each strain were fed standard chow and drinking water *ad libitum* and were housed in an AAALAC-accredited animal care facility at the West Virginia University Health Sciences Center and all protocols received prior IACUC approval. At 20 weeks of age, after an overnight fast, mice were anesthetized with injections of sodium pentobarbital (50 $mg \bullet kg^{-1}$ i.p.), and received tracheal intubation to facilitate maintenance of a patent airway. In all mice, a carotid artery was cannulated for determination of arterial pressure. Blood aliquots were drawn from the jugular vein cannula for determination of glucose and insulin (Linco), a lipid profile (Waco), and nitrotyrosine (Oxis).

Preparation of Isolated Skeletal Muscle Resistance Arterioles: In anesthetized mice, the intramuscular continuation of the right gracilis artery was removed and cannulated, as described previously (8). These first order arterioles were extended to their approximate *in situ* length and were equilibrated at 80% of the animal's mean arterial pressure in order to approximate the *in vivo* intralumenal pressure experienced by the animal (13). Following equilibration, arteriolar reactivity was evaluated in response to increasing concentrations of arachidonic acid (10^{-10} M – 10^{-6} M; Sigma). Additionally, in select experiments arteriolar reactivity was also evaluated in response to increasing concentrations of prostacyclin (PGI_2; 10^{-10} M – 10^{-6} M; Biomol) or carbocyclic thromboxane A_2 (TxA_2; 10^{-10} M – 10^{-6} M; Cayman).

Removal of the arteriolar endothelium was accomplished by passing an air bolus through the perfusate line into the isolated microvessel, the efficacy of which was determined from a loss of all dilator reactivity in response to application of 10^{-6} M acetylcholine (8). To assess the contribution of nitric oxide production or the generation of metabolites via cyclooxygenase as mediators of arteriolar reactivity, isolated vessels were treated with the nitric oxide synthase inhibitor L-N^G-nitroarginine methyl ester (L-NAME; 10^{-4} M for 45 minutes prior to agonist challenge; Sigma) or the cyclooxygenase antagonist indomethacin (INDO; 10^{-6} M for 60 minutes prior to agonist challenge; Sigma), respectively. To

determine the contribution of metabolites of arachidonic acid mediated via cytochrome P450 enzymes, vessels were treated with the suicide substrate inhibitor 17-octadecynoic acid (17-ODYA; 10^{-5} M for 60 minutes prior to agonist challenge; Sigma). Previous studies have demonstrated that 17-ODYA profoundly attenuates both the ω-hydroxylation (producing 20-hydroxyeicosatetraenoic acid; 20-HETE) and epoxygenation (producing epoxyeicosatrienoic acids; EETs) reactions of arachidonic acid through cytochrome P450 (24), thus preventing changes to vascular levels of 20-HETE or EETs as contributing mediators to endothelium-dependent dilation. To assess the contribution of lipoxygenase metabolites to the patterns of arteriolar dilation, vessels were treated with nordihydroguaiaretic acid (NDGA; 3×10^{-5} M for 45 minutes prior to agonist challenge; Biomol), a selective inhibitor of 12/15-lipoxygenases (20, 27). To antagonize vascular PGH_2/TxA_2 receptors, vessels were treated with SQ-29548 (10^{-5} M for 30 minutes prior to agonist challenge; Biomol), while inhibition of thromboxane synthase was accomplished using carboxyheptyl imidazole (CHI; 10^{-5} M for 45 minutes prior to agonist challenge; Biomol). To reduce vascular oxidant tone, arterioles were treated with 4-Hydroxy-2,2,6,6-tetramethylpiperidine-1-^{15}N-oxyl (TEMPOL; 10^{-4}M for 60 minutes prior to agonist challenge, Sigma).

Determination of Vascular Metabolites of Arachidonic Acid: Vascular production of 6-keto-prostaglandin $F_{1\alpha}$ (6-keto-$PGF_{1\alpha}$; the stable breakdown product of PGI_2; ref. 14), and 11-dehydro-thromboxane B_2 (11-dehydro-TxB_2; the stable plasma breakdown product of TxA_2; ref. 4) in response to challenge with arachidonic acid within the three mouse strains was assessed using pooled conduit arteries (femoral, saphenous, iliac, carotid arteries) from each mouse. Vessels were incubated in microcentrifuge tubes in 1 ml of physiological salt solution for 30 minutes under control conditions (21% O_2), after which time arachidonic acid (10^{-6} M) was added to the tube for an additional 30 minutes. After the second 30 minute period, the PSS was transferred to a new tube, frozen in liquid N_2 and stored at -80°C. Metabolite release by the vessels was determined using commercially available EIA kits for 6-keto-$PGF_{1\alpha}$ and 11-dehydro-TxB_2 (Cayman).

Data and Statistical Analyses: Active tone of individual arterioles at the equilibration pressure was calculated as $(\Delta D/D_{max})\bullet 100$, where ΔD is the diameter increase from rest in response to Ca^{2+}-free PSS, and D_{max} is the maximum diameter measured at the equilibration pressure in Ca^{2+}-free PSS.

Dilator responses of isolated arterioles following challenge with dilator agonists were fit with the three-parameter logistic equation:

$$y = \min + \left[\frac{\max - \min}{1 + 10^{\log ED_{50} - x}} \right]$$

where y represents the change in arteriolar diameter, "min" and "max" represent the lower and upper bounds, respectively, of the change in arteriolar diameter with increasing agonist concentration, x is the logarithm of the agonist concentration and $\log ED_{50}$ represents the logarithm of the agonist concentration (x) at which the response (y) is halfway between the lower and upper bounds.

Data are presented as mean±SEM. Statistically significant differences in measured and calculated parameters in the present study were determined using analysis of variance (ANOVA). In all cases, Student-Newman-Keuls post hoc test was used when appropriate and $p<0.05$ was taken to reflect statistical significance.

RESULTS

Table 1 presents baseline characteristics of the mouse groups in the present study. While all mice were of similar mass at 20 weeks of age, LDLR experienced a significant elevation in mean arterial pressure and fasting insulin concentration versus values in C57 or ApoE. Additionally, both ApoE and LDLR manifested a profound hypercholesterolemia, most severe in LDLR. Further, ApoE exhibited a significant hypertriglyceridemia as well, while plasma triglyceride levels in LDLR were not different from that in C57. Finally, plasma levels of nitrotyrosine, a marker of chronic elevations in oxidant stress, were significantly elevated in ApoE and LDLR as compared to C57. With regard to basal vascular tone, isolated arterioles from all mouse groups demonstrated a comparable resting active diameter and passive (calcium-free) diameter,

such that no significant difference in active tone was calculated between C57, ApoE and LDLR in the present study.

Data summarizing the dilator responses of skeletal muscle resistance arterioles from C57, ApoE and LDLR in response to challenge with increasing concentrations of arachidonic acid are presented in Figure 1. Under control conditions, the reactivity of arterioles from ApoE and LDLR, while not significantly different from each other, both demonstrated a reduction in their maximum bound as compared to responses in arterioles from C57. Endothelium-denudation via perfusion with an air bolus eliminated mechanical responses of vessels across the three strains in response to application of arachidonic acid.

The effects of pharmacological blockade of lipoxygenases and cyclooxygenases with NDGA and INDO, respectively, on arachidonic acid-induced vasodilation in isolated arterioles are summarized in Figure 2. In arterioles from C57 (Panel A), blockade of lipoxygenases with NDGA had no impact on dilator responses to arachidonic acid, while treatment with indomethacin abolished all dilation to arachidonic acid. Arterioles from ApoE, while demonstrating a blunted overall reactivity to arachidonic acid, also experienced a severe reduction in dilator reactivity following cyclooxygenase inhibition with indomethacin (Panel B). However, while treatment with NDGA alone did not impact arachidonic acid-induced dilation in vessels from ApoE, application of NDGA to vessels that had been treated with indomethacin eliminated the residual dilation in response to arachidonic acid that remained following cyclooxygenase inhibition alone. Finally, arterioles from LDLR appeared to demonstrate a dilator response to arachidonic acid challenge that was dependent on the production of metabolites generated via both lipoxygenases and cyclooxygenases, as antagonists to these pathways given in isolation resulted in modest reductions to the compromised level of reactivity, while treatment with both NDGA and indomethacin abolished all arachidonic acid-induced reactivity (Panel C). Treatment of isolated arterioles from C57, ApoE or LDLR with either L-NAME or 17-ODYA did not result in either significant or consistent effects of dilator responses following challenge with increasing concentrations of arachidonic acid (data not shown).

Figure 3 presents the effects of antagonizing thromboxane A_2 generation (with CHI) and action (with SQ-29548) on dilator responses of skeletal muscle arterioles in the present study. In control animals, application of either CHI or SQ-29548 had no impact on arteriolar dilation in response to increasing concentrations of arachidonic acid (Panel A). In contrast, arterioles from both ApoE (Panel B) and LDLR (Panel C) exhibited a significant improvement to their degree of arachidonic acid-induced dilation relative to untreated conditions following either inhibition of thromboxane synthase with CHI or blockade of the PGH_2/TxA_2 receptor (SQ-29548).

Data describing the arachidonic acid-induced generation of the cyclooxygenase products PGI_2 (estimated from levels of 6-keto-$PGF_{1\alpha}$) and TxA_2 (estimated from levels of 11-dehydro TxB_2) from pooled arteries of the three mouse groups in the present study are summarized in Figure 4. Following application of 10^{-6} M arachidonic acid, arteries from C57, ApoE and LDLR all demonstrated a significant increase in PGI_2 release, the degree of which was comparable between the three mouse strains (Panel A). In contrast, arachidonic acid-induced generation of TxA_2, while statistically significant in arteries from C57, demonstrated a substantially more robust response in vessels from both ApoE and LDLR (Panel B). Pre-treatment of pooled vessels with either CHI or indomethacin severely attenuated all arachidonic acid-induced TxA_2 generation in all three strains.

Arteriolar reactivity in response to challenge with prostacyclin (Panel A) or carbocyclic thromboxane A_2 (Panel B) in the three mouse groups is summarized in Figure 5. In response to increasing concentrations of prostacyclin, arterioles from C57 and ApoE demonstrated a very similar degree of dilator reactivity, although this response demonstrated a trend toward impairment in vessels from LDLR as compared to that in vessels from either other strain (Panel A). Arterioles from all three mouse strains exhibited very similar patterns of constrictor reactivity in response to challenge with increasing concentrations of carbocyclic thromboxane A_2 (Panel B).

Figure 6 presents the effects of treating vessels with the antioxidant TEMPOL, the thromboxane synthase inhibitor CHI, or both, on arteriolar responses to increasing concentrations of arachidonic acid.

Addition of TEMPOL did not have a significant impact on arteriolar diameter in vessels from any of the three mouse strains under resting conditions. In arterioles from C57 (Panel A), neither treatment with TEMPOL nor CHI had a significant impact on dilator reactivity to arachidonic acid. In contrast, for arterioles from both ApoE (Panel B) and LDLR (Panel C), treatment with either TEMPOL or CHI significantly improved dilator responses to arachidonic acid, with the effects of CHI being stronger than that for TEMPOL. Interestingly, in both ApoE and LDLR, combined treatment with CHI and TEMPOL did not have any effect on arachidonic acid-induced dilation beyond that determined for CHI treatment alone.

Figure 7 presents data describing the effects of treating arteries from C57, ApoE or LDLR with TEMPOL on arachidonic acid-induced thromboxane A_2 production. While treatment with the antioxidant had an insignificant impact on vascular thromboxane production in C57, incubation of vessels with TEMPOL significantly reduced the arachidonic acid-induced production of TxA_2 in both ApoE and LDLR. However, this reduction in thromboxane generation was only partial in nature, and levels of TxA_2 production in response to challenge with arachidonic acid following treatment with TEMPOL remained significantly increased versus that in untreated arteries from ApoE and LDLR.

DISCUSSION

Although hypercholesterolemia represents a powerful risk factor for the development of peripheral artery disease (1), the effects of hypercholesterolemia on vascular reactivity and endothelial function is less clearly understood. Given recent studies suggesting that diet-induced hypercholesterolemia can alter arachidonic acid metabolism and profoundly impact vascular reactivity through signaling mechanisms associated with the generation of thromboxane A_2 (15, 18, 21), the present study determined the effects of genetic hypercholesterolemia on the dilator reactivity of skeletal muscle resistance arterioles in response to challenge with arachidonic acid. More specifically, the hypothesis tested in this study was that arachidonic acid-induced arteriolar dilation in ApoE and LDLR would be impaired owing to either the production of, or vascular reactivity to, metabolites of arachidonic acid via cyclooxygenase, and that these alterations would be associated with an elevated oxidant stress.

Contrary to our results with dilator stimuli that are more strongly dependent on the bioavailability of endothelium-derived nitric oxide, where reactivity was largely maintained in the face of a profound reduction in this parameter (22), the results presented in Figure 1 indicate that skeletal muscle arteriolar dilation in response to increasing concentrations of arachidonic acid was significantly reduced in both ApoE and LDLR as compared to responses determined in C57. Interestingly, the data presented in this figure also strongly suggest that not only is the overwhelming majority of dilator reactivity in response to arachidonic acid dependent on a functional endothelium in control animals, the impairments to arteriolar dilation with this stimulus may also originate with alterations to endothelial function, rather than within vascular smooth muscle.

While results from the present study did not demonstrate a role for either nitric oxide bioavailability or for metabolites of arachidonic acid mediated via cytochrome P450 enzymes in terms of contributing to the arachidonic acid-induced dilator reactivity of skeletal muscle arterioles in any of the three mouse strains, activity mediated through cyclooxygenase (and to a lesser extent lipoxygenase) were critical. While arteriolar dilation in response to arachidonic acid was mediated entirely via cyclooxygenase in vessels from C57, vessels from ApoE and LDLR demonstrated a dilator response that was increasingly a function of metabolites via both cyclooxygenase and lipoxygenase, with this effect being more pronounced in LDLR than in ApoE, where the response was still predominantly cyclooxygenase-dependent. However, the data presented in Figure 2 do not provide significant insight into the impaired dilator reactivity demonstrated in arterioles of ApoE and LDLR in response to challenge with arachidonic acid beyond the critical involvement of cyclooxygenase. Given previous studies suggesting that the development of the hypercholesterolemic condition can profoundly impact arachidonic acid metabolism in general (7, 18), and the recent studies from both Pfister (15) and Srisawat *et al.* (21) that implicate altered behavior mediated through thromboxane generation/action as contributing mechanism to altered patterns of vascular reactivity with hypercholesterolemia, we treated vessels from ApoE and LDLR with an inhibitor of thromboxane synthase (CHI) or an antagonist for the PGH_2/TxA_2 receptor (SQ-29548). As shown in Figure 3, while neither of these

agents had a significant role in the dilator responses in arterioles from C57, application of either CHI or SQ-29548 resulted in a significant improvement in the dilator responses of arterioles from ApoE or LDLR in response to challenge with increasing concentrations of arachidonic acid. Interestingly, the ameliorative effect was comparable with either pharmacological agent. While this implicates either increased thromboxane generation or an increased gain/sensitivity at the vascular thromboxane receptor as contributing mechanisms to the impaired arachidonic acid-induced arteriolar dilation, these data do not provide insight into which component may be most responsible. However, these data do strongly suggest that the development of a thromboxane-sensitive component which may act to constrain arachidonic acid-induced arteriolar dilation accompanies the evolution of genetic hypercholesterolemia.

As both CHI and SQ-29548 elicited similar improvements to arteriolar dilation in response to arachidonic acid challenge in ApoE and LDLR, it was necessary to discern which processes contributed to the constrained dilator reactivity: 1) increasing thromboxane A_2 production in response to arachidonic acid production, 2) increased vascular reactivity to produced thromboxane A_2, or both. The data presented in Figure 4 indicate that arachidonic acid-induced generation of PGI_2 (estimated from 6-keto-$PGF_{1\alpha}$ levels) remained intact in arteries of ApoE and LDLR as compared to that determined in C57, an observation that is consistent with previous studies in the coronary vasculature of ApoE mice (9). In contrast, arachidonic-acid induced generation of thromboxane A_2 (estimated from 11-dehydro-TxB_2 levels) was significantly increased with the evolution of genetic hypercholesterolemia in ApoE and LDLR. When taken together with data in Figure 5, which suggest that the sensitivity of resistance arterioles from ApoE and LDLR in response to increasing concentrations of either prostacyclin or thromboxane A_2 is not dramatically altered from that determined for C57 control mice, these data may provide compelling evidence that a predominant contributing mechanism underlying the constrained arteriolar dilation with increasing concentrations of arachidonic acid may be the development of an increased generation of the constrictor prostanoid thromboxane A_2, which antagonizes the dilator effects associated with the generation of prostacyclin.

Given that previous studies have clearly demonstrated the critical role for elevated oxidant tone in the increased generation of thromboxane through cyclooxygenase in response to challenge with arachidonic acid (2, 28, 29), and our observations of an increase in the plasma levels of nitrotyrosine in the ApoE and LDLR as compared to that determined in C57, the data presented in Figures 6 and 7 provide some insight into the potential role that elevated vascular oxidant tone may play in the increased arachidonic acid-induced thromboxane A_2 generation with genetic dyslipidemia. While treatment with TEMPOL had no impact on arachidonic acid-induced dilation or TxA_2 generation in arterioles from C57, it significantly improved the dilator response in microvessels from both ApoE and LDLR and reduced the levels of TxA_2 production. However, in vessels from both strains, this improvement in dilator reactivity following treatment with the antioxidant was less pronounced than that determined following treatment with the inhibitor of thromboxane synthase, CHI. Further, combined treatment with both TEMPOL and CHI did not result in an improvement beyond that determined with CHI treatment alone. Additionally, while pre-treatment of pooled vessels with TEMPOL lowered arterial thromboxane production in response to challenge with arachidonic acid, the levels of thromboxane production remained significantly elevated despite the addition of the antioxidant. Taken together these results suggest that, while an enhanced arachidonic acid-induced genesis of thromboxane A_2 via thromboxane synthase represents a strong contributor to the constrained dilator reactivity in skeletal muscle arterioles of ApoE and LDLR mice, the presence of an elevated vascular oxidant tone may represent a partial contributor to this shift in the metabolism of arachidonic acid. Clearly, these results suggest that other parameters, independent of acute changes in vascular oxidant tone, also contribute to this increased generation of thromboxane A_2. Potential avenues for ongoing investigation in this regard can include the study of not only the effects of chronic elevations in vascular oxidant tone, but also the progression of a chronic state of inflammation associated with dyslipidemia (10, 26) and how these processes can ultimately impact pathways of arachidonic acid metabolism.

In summary, with the development of genetic hypercholesterolemia in ApoE and LDLR mice, the dilator reactivity of skeletal muscle resistance arterioles in response to increasing concentrations of

arachidonic acid is impaired. This impairment does not appear to be associated with a reduction in the generation/release of, or an altered arteriolar reactivity to, prostacyclin. However, with the evolution of this dyslipidemic condition, there appears to be an increase in the arachidonic acid-induced generation of the vasoconstrictor metabolite thromboxane A_2. While there does not appear to be an alteration to the arteriolar constrictor reactivity to thromboxane, the increased generation of this metabolite may compete with the dilator effects of prostacyclin, thus limiting net dilator reactivity in response to arachidonic acid. Further, while an increase in vascular oxidant stress appears to contribute to this response, additional mechanisms which are independent of acute alterations to oxidant tone also contribute to this effect. Future investigation will be required to discern which mechanistic alterations associated with the development of hypercholesterolemia contribute to the increased production of thromboxane A_2, and what the implications of this shift in the metabolism of arachidonic acid are for issues such as the integrated control of tissue perfusion, tissue oxygenation and the protection from atherogenesis and atherothrombosis.

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Hypercholesterolemia, thromboxane, and arteriolar reactivity

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Table 1. Baseline characteristics of mice and individual arterioles used in the present study. * $p<0.05$ vs. C57; † $p<0.05$ vs. ApoE.

	C57	**ApoE**	**LDLR**
Mass (g)	33±2	34±2	33±2
MAP (mmHg)	88±4	92±3	106±5*†
$[Glucose]_{blood}$ (mg/dl)	84±7	103±11	115±7*
$[Insulin]_{plasma}$ (ng/ml)	1.1±0.3	1.6±0.3	2.8±0.5*
$[Total\ Cholesterol]_{plasma}$ (mg/dl)	88±9	288±17*	364±22*†
$[LDL\ Cholesterol]_{plasma}$ (mg/dl)	49±5	260±11*	338±19*†
$[Triglycerides]_{plasma}$ (mg/dl)	88±10	175±14*	116±18†
$Nitrotyrosine_{plasma}$ (ng/ml)	14±5	54±11*	60±14*
Inner Diameter – Active (μm)	54±4	55±5	51±4
Inner Diameter – Passive (μm)	128±5	122±4	118±7
Active Tone (%)	57±3	55±4	56±4

FIGURE LEGENDS

Figure 1. Data describing the dilator reactivity of isolated skeletal muscle resistance arterioles of C57, ApoE and LDLR mice in response to increasing concentrations of arachidonic acid. Data, presented as mean±SEM, are shown for arterioles under control conditions and following removal of the vascular endothelium using air bolus perfusion (please see text for details). n=6 animals for each strain; * $p<0.05$ vs. C57; † $p<0.05$ vs. control within that strain.

Figure 2. Data describing the dilator responses of isolated skeletal muscle resistance arterioles of C57 (Panel A), ApoE (Panel B) and LDLR (Panel C) mice in response to increasing concentrations of arachidonic acid. Data, presented as mean±SEM, are shown for arterioles under control conditions, and following pharmacological inhibition of cyclooxygenases with indomethacin, lipoxygenases with NDGA or combined inhibition of both enzymatic pathways (please see text for details). n=5-10 animals for each group; * $p<0.05$ vs. control conditions, † $p<0.05$ vs. no response.

Figure 3. Data describing the dilator responses of isolated skeletal muscle resistance arterioles of C57 (Panel A), ApoE (Panel B) and LDLR (Panel C) mice in response to increasing concentrations of arachidonic acid. Data, presented as mean±SEM, are shown for arterioles under control conditions, and following pharmacological inhibition of PGH_2/TxA_2 receptors with SQ-29548 and thromboxane synthase with CHI (please see text for details). n=6-7 animals for each group; * $p<0.05$ vs. control conditions.

Figure 4. Data describing the arterial production of prostacyclin (as 6-keto-$PGF_{1\alpha}$; Panel A) or thromboxane A_2 (as 11-dehydro TxB_2; Panel B) from C57, ApoE and LDLR in response to 10^{-6} M arachidonic acid. Data, presented as mean±SEM, are shown under control conditions, and following pharmacological inhibition of cyclooxygenase with indomethacin or thromboxane synthase (with CHI), as appropriate. n=8 animals for

each group, with each n representing pooled arteries from an individual mouse; please see text for details. * $p<0.05$ vs. respective control; † $p<0.05$ vs. C57 under that condition; ‡ vs. ApoE under that condition.

Figure 5. Data (mean±SEM) describing the reactivity of isolated skeletal muscle resistance arterioles of C57, ApoE and LDLR mice in response to increasing concentrations of prostacyclin (Panel A) or carbocyclic thromboxane A_2 (Panel B). n=6 animals for each group, no significant differences were identified in the vascular reactivity in response to increasing concentrations of prostacyclin or thromboxane A_2.

Figure 6. Data, presented as mean±SEM, describing the dilator responses of isolated skeletal muscle resistance arterioles of C57 (Panel A), ApoE (Panel B) and LDLR (Panel C) mice in response to increasing concentrations of arachidonic acid. Data are shown for arterioles under control conditions, following treatment of vessels with the antioxidant TEMPOL, following pharmacological inhibition of thromboxane synthase with CHI, and following treatment with both TEMPOL and CHI. n=8-10 animals for each group; * $p<0.05$ vs. control conditions; † $p<0.05$ vs. treatment with TEMPOL alone.

Figure 7. Data describing the arterial production of thromboxane A_2 (as 11-dehydro TxB_2; Panel B) from C57, ApoE and LDLR in response to 10^{-6} M arachidonic acid. Data, presented as mean±SEM, are shown under control conditions, and following treatment of pooled arteries with the antioxidant TEMPOL (10^{-4} M). n=6 animals for each group, with each n representing pooled arteries from an individual mouse; please see text for details. * $p<0.05$ vs. within-strain/no arachidonic acid; † $p<0.05$ vs. within-strain/with arachidonic acid.

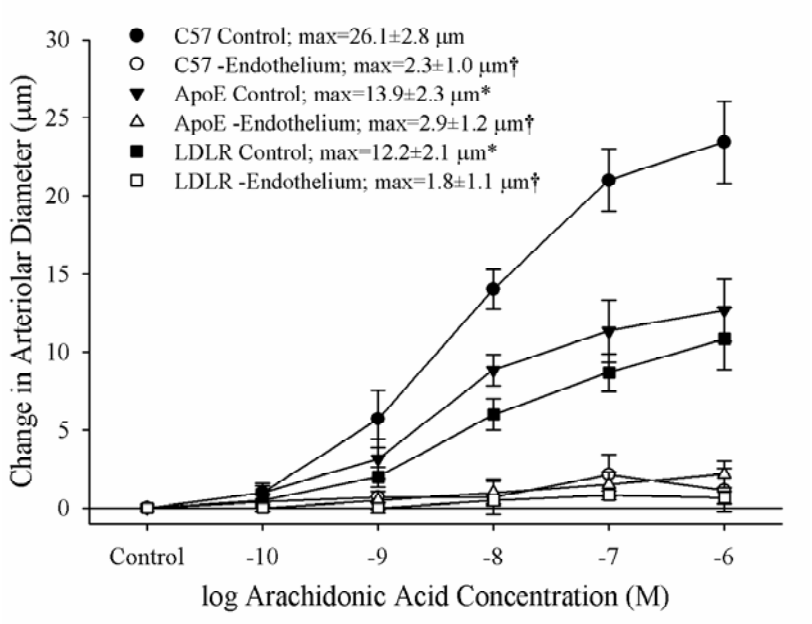
C57 Control; max=26.1±2.8 µm
C57 -Endothelium; max=2.3±1.0 µm†
ApoE Control; max=13.9±2.3 µm*
ApoE -Endothelium; max=2.9±1.2 µm†
LDLR Control; max=12.2±2.1 µm*
LDLR -Endothelium; max=1.8±1.1 µm†
Change in Arteriolar Diameter (µm)
30
25
20
15
10
5
0
Control
-10
-9
-8
-7
-6
log Arachidonic Acid Concentration (M)

Figure 1

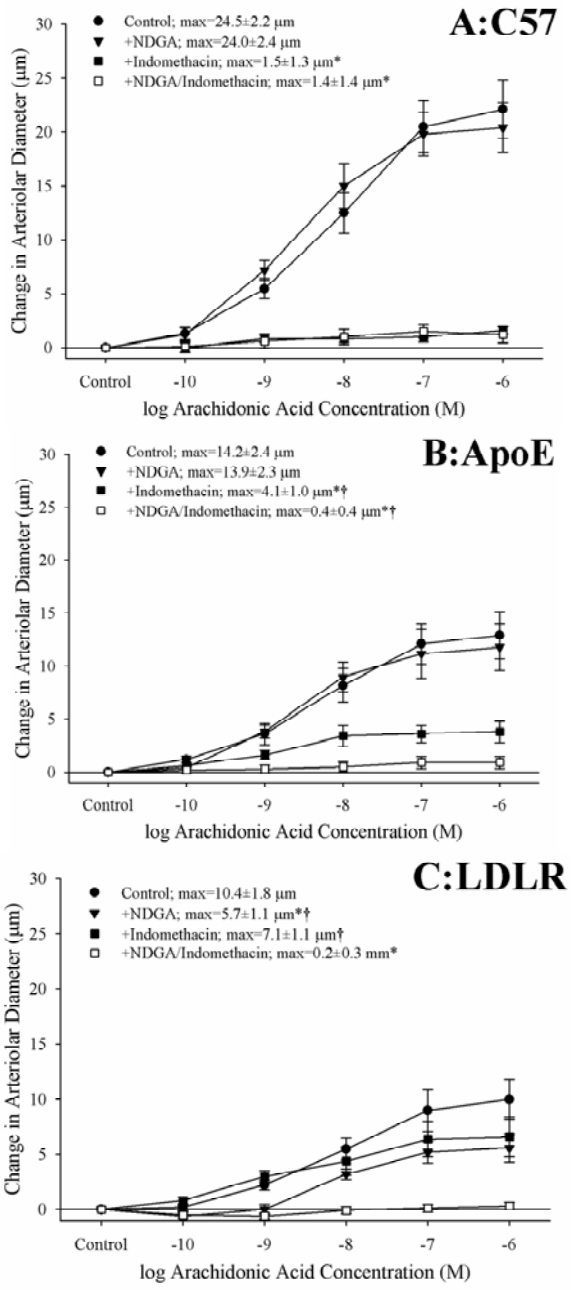
A:C57
Control; max=24.5±2.2 µm
+NDGA; max=24.0±2.4 µm
+Indomethacin; max=1.5±1.3 µm*
+NDGA/Indomethacin; max=1.4±1.4 µm*
Change in Arteriolar Diameter (µm)
log Arachidonic Acid Concentration (M)
B:ApoE
Control; max=14.2±2.4 µm
+NDGA; max=13.9±2.3 µm
+Indomethacin; max=4.1±1.0 µm*†
+NDGA/Indomethacin; max=0.4±0.4 µm*†
Change in Arteriolar Diameter (µm)
log Arachidonic Acid Concentration (M)
C:LDLR
Control; max=10.4±1.8 µm
+NDGA; max=5.7±1.1 µm*†
+Indomethacin; max=7.1±1.1 µm†
+NDGA/Indomethacin; max=0.2±0.3 mm*
Change in Arteriolar Diameter (µm)
log Arachidonic Acid Concentration (M)

Figure 2

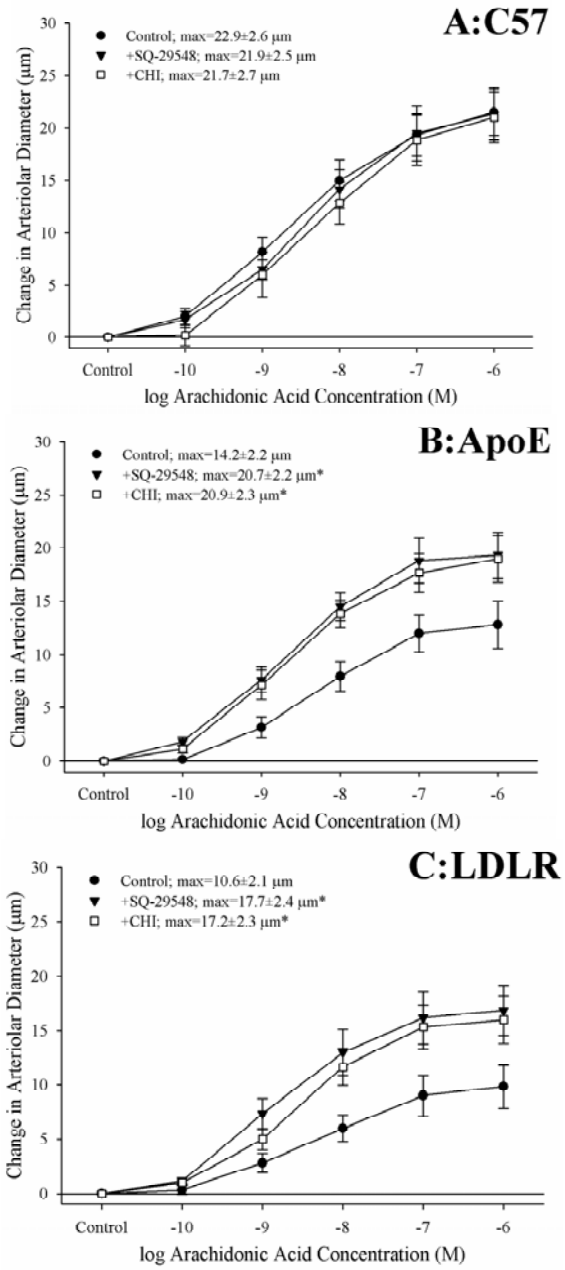
A:C57
Control; max=22.9±2.6 µm
+SQ-29548; max=21.9±2.5 µm
+CHI; max=21.7±2.7 µm
Change in Arteriolar Diameter (µm)
log Arachidonic Acid Concentration (M)
B:ApoE
Control; max=14.2±2.2 µm
+SQ-29548; max=20.7±2.2 µm*
+CHI; max=20.9±2.3 µm*
C:LDLR
Control; max=10.6±2.1 µm
+SQ-29548; max=17.7±2.4 µm*
+CHI; max=17.2±2.3 µm*
Control
-10
-9
-8
-7
-6

Figure 3

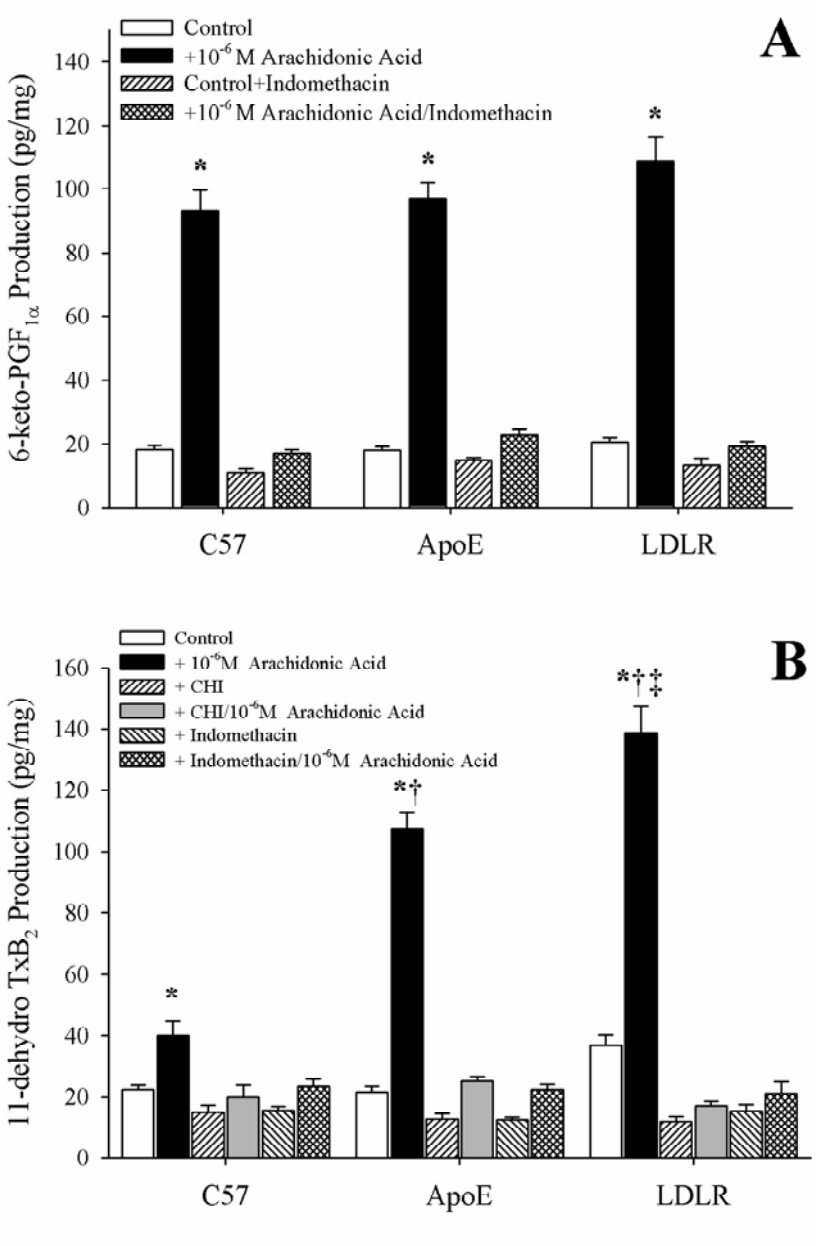
A
Control
+10-6 M Arachidonic Acid
Control+Indomethacin
+10-6 M Arachidonic Acid/Indomethacin
6-keto-PGF1α Production (pg/mg)
0
20
40
60
80
100
120
140
*
*
*
C57
ApoE
LDLR
B
Control
+ 10-6M Arachidonic Acid
+ CHI
+ CHI/10-6M Arachidonic Acid
+ Indomethacin
+ Indomethacin/10-6M Arachidonic Acid
11-dehydro TxB2 Production (pg/mg)
0
20
40
60
80
100
120
140
160
*
*†
*†‡
C57
ApoE
LDLR

Figure 4

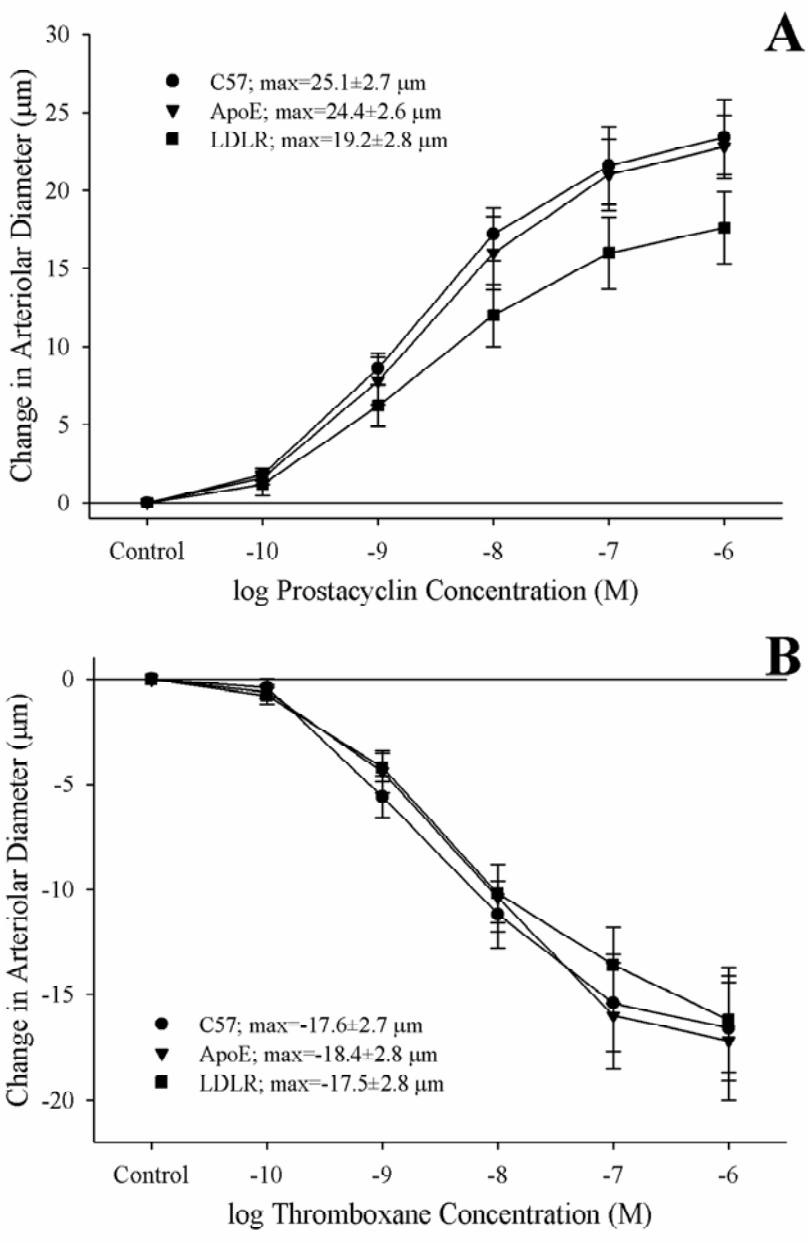
A
C57; max=25.1±2.7 µm
ApoE; max=24.4±2.6 µm
LDLR; max=19.2±2.8 µm
Change in Arteriolar Diameter (µm)
30
25
20
15
10
5
0
Control
-10
-9
-8
-7
-6
log Prostacyclin Concentration (M)
B
Change in Arteriolar Diameter (µm)
0
-5
-10
-15
-20
C57; max=-17.6±2.7 µm
ApoE; max=-18.4±2.8 µm
LDLR; max=-17.5±2.8 µm
Control
-10
-9
-8
-7
-6
log Thromboxane Concentration (M)

Figure 5

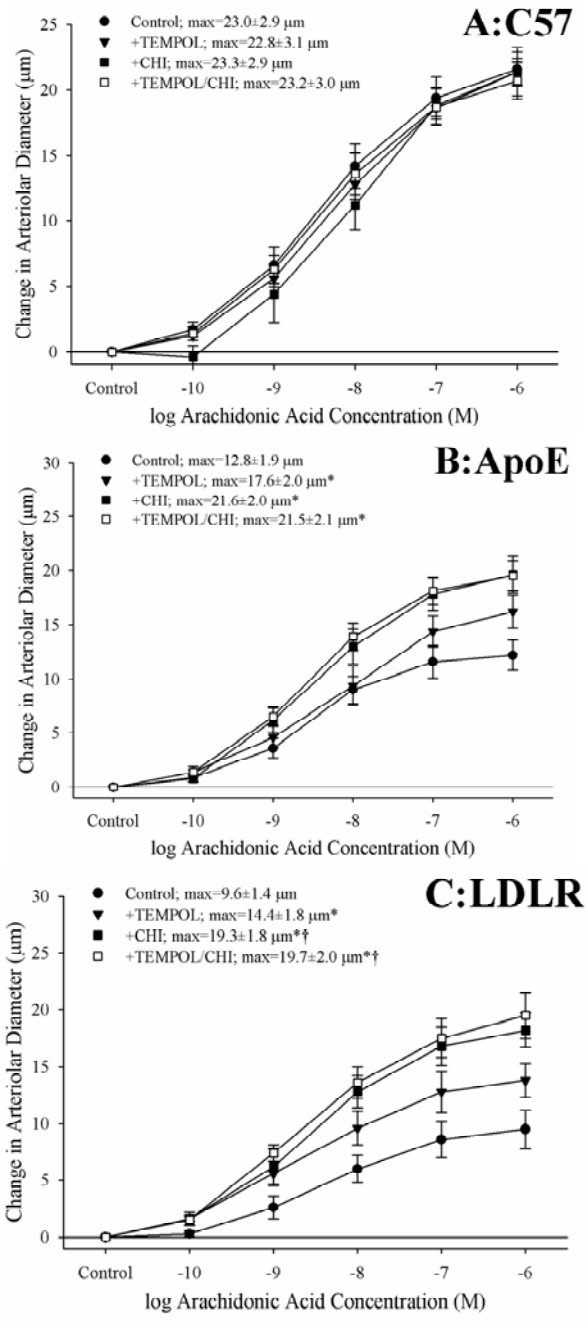
A:C57
Control; max=23.0±2.9 µm
+TEMPOL; max=22.8±3.1 µm
+CHI; max=23.3±2.9 µm
+TEMPOL/CHI; max=23.2±3.0 µm
Change in Arteriolar Diameter (µm)
log Arachidonic Acid Concentration (M)
B:ApoE
Control; max=12.8±1.9 µm
+TEMPOL; max=17.6±2.0 µm*
+CHI; max=21.6±2.0 µm*
+TEMPOL/CHI; max=21.5±2.1 µm*
C:LDLR
Control; max=9.6±1.4 µm
+TEMPOL; max=14.4±1.8 µm*
+CHI; max=19.3±1.8 µm*†
+TEMPOL/CHI; max=19.7±2.0 µm*†

Figure 6

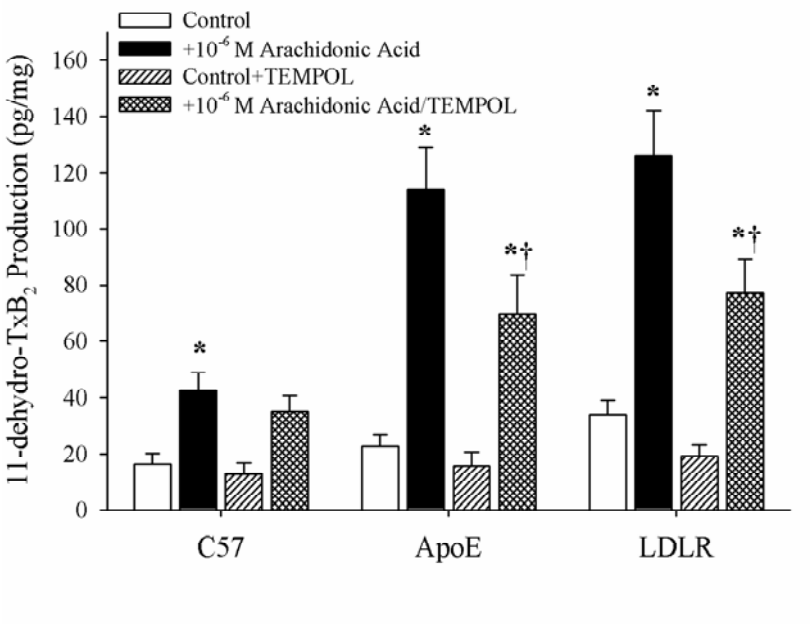
Control
+10-6 M Arachidonic Acid
Control+TEMPOL
+10-6 M Arachidonic Acid/TEMPOL
11-dehydro-TxB2 Production (pg/mg)
0
20
40
60
80
100
120
140
160
*
*
*†
*
*†
C57
ApoE
LDLR

Figure 7

IMPAIRED SKELETAL MUSCLE ARTERIAL ENDOTHELIUM-DEPENDENT DILATION IN FAMILIAL HYPERCHOLESTEROLEMIA: IMPACT OF CHRONIC EXERCISE AND ANTI-CHOLESTEROL THERAPIES

Phoebe A. Stapleton[1,3], Adam. G. Goodwill[2,3], Milinda E. James[2,3] and Jefferson C. Frisbee[2,3]

Division of Exercise Physiology[1], Department of Physiology and Pharmacology[2] and
Center for Cardiovascular and Respiratory Sciences[3]
West Virginia University School of Medicine, Morgantown, WV 26506

Running Head: Interventional Strategies in Hypercholesterolemia
Key Words: skeletal muscle microcirculation, endothelium-dependent dilation, vascular reactivity, mouse models of cardiovascular disease

Send Correspondence to:

Jefferson C. Frisbee, Ph.D.
Center for Cardiovascular and Respiratory Sciences
Department of Physiology and Pharmacology
Robert C. Byrd Health Sciences Center
PO Box 9105
West Virginia University School of Medicine
Morgantown, WV 26505
Phone: (304) 293-6527
Fax: (304) 293-5513
Email: jfrisbee@hsc.wvu.edu

ABSTRACT

Hypercholesterolemia is defined as excessively high plasma cholesterol levels, and is a strong risk factor for many negative cardiovascular events. Total cholesterol levels above 200 mg/dl have repeatedly been correlated as an independent risk factor for development of peripheral vascular (PVD) and coronary artery disease (CAD). With specific relevance to the microcirculation, it has been clearly demonstrated that evolution of hypercholesterolemia is associated with endothelial cell dysfunction, a near-complete abrogation in vascular nitric oxide bioavailability, elevated oxidant stress, and the creation of a strongly pro-inflammatory condition; symptoms which can culminate in profound impairments/alterations to vascular reactivity. Considerable attention has been directed toward evaluating mechanisms and compensatory mechanisms by which hypercholesterolemia may impair microvascular endothelium dependent dilation. Clinically relevant pharmaceutical treatment options include therapies which decrease circulating cholesterol by preventing either formation in the liver or absorption in the intestine also have pleiotropic effects with may directly improve peripheral vascular outcomes. Physical activity is known to decrease PVD/CAD risk factors, increase circulating levels of high density lipoprotein and improve both cardiac and vascular function. The mechanistic consequences of these treatments are not well understood within the realms of inflammation, oxidative stress, and vascular reactivity; therefore, the hypothesis of this study was to determine the effects of pharmaceutical treatment, exercise, and combined therapy to improve restore endothelium dependent microvascular reactivity, NO bioavailability, decrease in oxidant stress, and decrease the a pro-inflammatory phenotype within the condition of familial hypercholesterolemia.

Arterioles from the low density lipoprotein receptor (LDLR) gene deletion male mouse models of hypercholesterolemia at 20 weeks of age show no significant improvement to any of the ameliorative therapies. Interestingly, the pharmaceutical only group shows a detriment to the endothelium dependent

dilation within the C57/Bl/6J (C57) controls, with no significant improvements to the anti-inflammatory or anti-oxidant profiles. While the exercise and combination therapy were able to improve the overall inflammatory and oxidant profiles with respect to CRP, MCP-1, RANTES, and nitrotyrosine. These results suggest that independent of any cholesterol lowering effects, within a normocholesterolemic population, prophylactic administration of cholesterol lowering pharmaceutical therapies may be detrimental to the endothelium dependent vascular reactivity. Additionally, exercise or combination therapy may provide the greatest benefit, due to the overall improvements in the anti-inflammatory and anti-oxidant profiles.

INTRODUCTION

As high total cholesterol levels are considered to be a major independent risk factor for development of peripheral vascular disease (PVD) and coronary artery disease (CAD), considerable attention has been directed toward evaluating the impact and mechanisms of cholesterol lowering therapies and alternative interventions for cardiovascular outcomes (10; 34). Data acquired by the National Health and Nutrition Examination Survey 2005–2006 found that the mean total serum cholesterol level for Americans, over the age of 20, was 199 mg/dL, just below the American Heart Association (AHA) recommended level of 200 mg/dL (1; 22). Unfortunately, 16% of adults were found to have total cholesterol levels of more than 240 mg/dL, this level is considered by the AHA to carry twice the cardiovascular risk of those individuals at the desired level (1; 22).

With specific relevance to the microcirculation, it has been clearly demonstrated that hypercholesterolemia is associated with endothelial cell dysfunction, decreased nitric oxide bioavailability, elevated oxidant stress, and a strongly pro-inflammatory condition; symptoms which can culminate in profound impairments to vascular reactivity. Previous studies in our laboratory have suggested that the signaling mechanisms responsible for dilator reactivity of skeletal muscle resistance

arterioles in hypercholesterolemic animals remains intact however altered due to a decreased availability of NO compared to control (16; 25; 37; 39; 42; 43; 50). This reduction in the availability of NO within the hypercholesterolemic vasculature either by deficits in production (i.e. NOS inhibition) or due to increases in NO degradation (i.e. NO scavenge). With the progression of familial hypercholesterolemia there is a greater reliance on the arachidonic acid dilator metabolites via 12- and 15-lipoxygenase to maintain endothelium dependent reactivity (39). Additionally, with the evolution of hypercholesterolemia, an increase in vascular oxidant stress leading to alterations in arachidonic acid metabolism has also been reported (12).

Investigation into vascular consequences of chronic hypercholesterolemia, the mechanisms through which this occurs, and the potentially beneficial effects of ameliorative therapies have received considerable attention in recent years. Pharmaceutical therapies are currently the most commonly prescribed medication within the United States. Statins drugs can act to decrease cholesterol levels by preventing the formation of cholesterol in the liver or preventing the absorption of cholesterol via the intestine, while ezetimibe acts selectively to inhibit the absorption of cholesterol from the intestine (38). These drugs, when used in combination (ezetimibe/simvastatin; Vytorin) act together via complementary pathways to prevent cholesterol absorption from the intestine and hepatic production in patients with hypercholesterolemia. Long term co-administration of these drugs have been shown to reduce LDL blood cholesterol levels by 60% while concurrently raising HDL levels and limiting liver toxicity traditionally caused by statin treatment alone (6; 32).

These are generally prescribed for the cholesterol lowering effects; however reports of additional independent pleiotropic effects include, but are not limited to reducing inflammation, decreasing ROS, and increasing NO bioavailability. These pharmaceutical agents have been found to independently

improve endothelial function with respect to FMD, with 150% in normocholesterolemic and 180% in hypercholesterolemic patients who had also been diagnosed with peripheral artery disease (13).

The AHA and American College of Sports Medicine (ACSM) have recently released joint guidelines recommending aerobic and resistance physical activities for individuals under the age of 65 to maintain health, reduce risk of chronic disease states, and manage current risk factors including hypercholesterolemia (14; 20; 40). Hypercholesterolemia has been shown to impair aerobic capacity by impairing dilator regulation, thought to be due to a lack of vascular reactivity stemming from a reduction in NO bioavailability (24). However acute and chronic exercise programs have been shown to improve vascular reactivity, reduce pro-inflammatory phenotypes, and improve the oxidant balance (5; 16; 28; 30).

However, the effects of these ameliorative therapies are not mechanistically well understood. Therefore, the purpose of the present study was to evaluate the vascular reactivity and determine the capacity of pharmaceutical treatment, exercise, and combined therapy to restore NO bioavailability, decrease in oxidant stress, and decrease the a pro-inflammatory phenotype within the condition of familial hypercholesterolemia.

MATERIALS AND METHODS

Animals: The present study used two strains of mice, the C57/Bl/6J (C57) as the control strain and the low density lipoprotein receptor gene deletion (B6.129S7-*Ldlr*^{tm1Her}/J; LDLr) mice on the C57/Bl/6J background. All mice were purchased from Jackson Laboratories (Bar Harbor, ME) at 8 weeks of age. The LDLr gene deletion mouse is a model of human familial hypercholesterolemia, manifesting a profound increase in serum LDL levels while ingesting a normal diet (19).

Male mice of each strain were fed standard chow and drinking water *ad libitum* and were housed the animal care facility at the West Virginia University Health Sciences Center and all protocols received

prior IACUC approval. At approximately 21 weeks of age, mice were anesthetized with injections of sodium pentobarbital (50 mg•kg^{-1} i.p.), and a carotid artery was cannulated for determination of arterial pressure. Blood aliquots were drawn from the carotid artery cannula for determination lipid profile levels (Stanbio), nitrotyrosine (Upstate), TNF-α (Invitrogen), and multiplex cytokine and chemokine levels (Millipore). At this age, the degree of the microvascular dysfunction was not so severe that it would not be amenable to amelioration via interventional strategies. Thus, the use of this age range allows us to examine the efficacy of interventional strategies for improving microvascular outcomes. The gastrocnemius complex was also removed and snap frozen in liquid N_2 immediately after extraction, these were then used to run citrate synthase assays, following the manufacturer protocol (Sigma).

Interventions: At 10 weeks of age, 10 mice from each strain (C57 and LDLr) were placed into one of four groups: control, exercise, pharmaceutical treatment, or combined exercise and pharmaceutical therapies.

Swimming Protocol: The exercise and combination groups participated in swimming twice each day separated by a 4-hour rest, 5-days a week for 5-weeks. The mice were acclimated to the swim training in 30°C – 34°C water starting with 10-minute sessions increasing daily by 10 minutes until reaching the plateau at 90-minutes each session for the remainder of the 5 weeks. This protocol has been shown to develop cardiac hypertrophy in normal animals and to minimize and plateau obesity, hyperlipidemia, and hypercholesterolemia in db/db mice (9; 27).

Pharmaceutical Treatment: The pharmaceutical and combination groups received a combination of 1mg/kg/day of ezetimibe (Zetia) and 50 mg/kg/day of simvastatin via chow for 10-weeks, beginning at 10 weeks of age. Chow was weighed, recorded, and replaced 2x per week.

Preparation of Isolated Skeletal Muscle Resistance Arterioles: In anesthetized mice, the intramuscular continuation of the right gracilis artery was surgically removed and cannulated, as described previously

for rats (14). These first order arterioles were extended to their approximate *in situ* length and were equilibrated at 80% of the animal's mean arterial pressure in order to approximate the *in vivo* intralumenal pressure experienced by the animal (26). Following equilibration, arteriolar reactivity was evaluated randomly in response to: 1) hypoxia; a reduction in superfusate and perfusate PO_2 from ~135 mmHg (21% O_2) to ~40 mmHg (0% O_2), 2) acetylcholine (10^{-10} M – 10^{-6} M; Sigma), 3) DetaNONOate (10^{-7} M – 10^{-4} M; Sigma) and 4) thromboxane mimetic U-46619 (10^{-12} M – 10^{-8} M; Biomol). To assess the contribution of NO production or the generation of metabolites via cyclooxygenase as mediators of arteriolar dilator reactivity, isolated vessels were treated with the NO synthase inhibitor L-N^G-nitroarginine methyl ester (L-NAME; 10^{-5} M; Sigma) or the cyclooxygenase antagonist indomethacin (INDO; 10^{-6} M; Sigma), respectively. Following assessments of arteriolar reactivity, the perfusate and superfusate were replaced with Ca^{2+}-free PSS and vessels were treated with 10^{-6} M adenosine to determine maximum dilation.

Multiplex Analysis: Whole blood was collected from live animals directly into K_2 EDTA containing tubes and immediately centrifuged for 10 minutes at 2500 x g. The plasma layer (supernatant) was then transferred into 1.5mL microcentrifuge tubes. These tubes were snap frozen in liquid N_2 before being placed at -80°C for long term storage (< 6 months). Samples were slowly thawed and placed on ice. Analysis was performed using multiplex suspension bead array immunoassays on a Luminex 200 system (Luminex Corporation; Austin, TX). The levels of cytokines were measured using Mouse Cytokine, Mouse CVD panels, Mouse CRP Single Plex, and Mouse Soluble Cytokine Receptor panels (Millipore; St. Charles, MO) for ICAM, IL-1B, IL-6, IL-10, MCP-1, MMP9, RANTES, sVCAM-1, sICAM-1, and e-selectin. Samples were diluted following manufacturer's recommendations and assayed in duplicate in accordance with manufacturer's instructions. In brief, plates were blocked, after which assay buffer, samples and an antibody immobilized bead cocktail were added to each well. The plates

were then kept in the dark for all steps subsequent to bead cocktail addition to minimize the effects of photobleaching. Plates were incubated for 18-20 hours at 4°C with agitation, washed, and treated with detection antibody. Following 2-hour room temperature incubation with agitation, strepavidin-phycoerythrin was added to the wells and final 30 minute room temperature incubation with agitation performed. Wells were thoroughly washed, beads resuspended in sheath fluid, and the plate was analyzed. Concentrations of each cytokine were calculated based on a standard curve generated on the same plate and using a 5-parameter logistic fit analysis on Luminex 100 IS software version 2.3.182 (Luminex Corporation; Austin, TX).

Western Blot Analysis: Pooled isolated non-aortic conduit vessels were snap frozen in liquid N_2 immediately after extraction. Protein was harvested from by repeated vortexing and boiling in a sample buffer containing 0.045M Tris-Cl pH 6.8, 10% by volume glycerol, 1% by volume SDS, 0.05M dithiothreotol, and 1% by volume 2-Mercaptoethanol, however to detect phosphorylation eNOS species, eNOS affinity purified from cell lysates using 2',5'- ADP-Sepharose (Pharmacia). Protein for eNOS phosphorylation will be size fractionated by SDS-PAGE. The total protein concentration of each sample was determined using a Nano-Orange assay (Invitrogen, California) according to the manufacturer's protocol. For each gel, protein samples were diluted in sample buffer to an equal concentration, boiled for 10 min and spun for 10 min at 9300 x g prior to loading onto precast 10% Bis-Tris polyacrylamide gels (Invitrogen, California). Gels were loaded with 50ug of total protein per well. Electrophoresis was be carried out at 150V for 1.5 h and resolved proteins were transferred to an Immobilon PVDF membrane (Millipore, Massachusetts) at 30V for 1.25 hours using Invitrogen's Sure Lock Mini-Cell electrophoresis system and associated X-Cell II Blot Module. Membranes were blocked overnight at 4°C in Superblock T-20 (Pierce Biotechnology, Illinois) and then incubated with associated antibodies eNOS (B&D) or ph-eNOS (Zymed) for an additional 1.5 hours. Membranes were then washed and

incubated with appropriate horseradish peroxidase conjugated secondary antibody. Amersham ECL western blotting kits were used to visualize proteins. Membranes were subsequently washed and incubated with GAPDH-HRP antibody for 1 hour, washed, visualized using an Amersham ECL western blotting kit, and measured using densitometry (11).

Data and Statistical Analyses: Active tone of individual arterioles at the equilibration pressure was calculated as $(\Delta D/D_{max})\bullet 100$, where ΔD is the diameter increase from rest in response to Ca^{2+}-free PSS, and D_{max} is the maximum diameter measured at the equilibration pressure in Ca^{2+}-free PSS.

Dilator responses of isolated arterioles following challenge with dilator agonists were fit with the four-parameter logistic equation:

$$y = \min + \left[\frac{\max - \min}{1 + 10^{\log ED_{50} - x}} \right]$$

where y represents the change in arteriolar diameter, "min" and "max" represent the lower and upper bounds, respectively, of the change in arteriolar diameter with increasing agonist concentration, x is the logarithm of the agonist concentration and $\log ED_{50}$ represents the logarithm of the agonist concentration (x) at which the response (y) is halfway between the lower and upper bounds.

Data are presented as mean±SEM. Statistically significant differences in measured and calculated parameters in the present study were determined using analysis of variance (ANOVA). In all cases, Holm-Sidak test was used when appropriate and $p<0.05$ was taken to reflect statistical significance.

RESULTS

Table 1 presents baseline characteristics of the eight mouse groups used in the present study. While all mice were of comparable MAP at 20 weeks of age, the mice in each of the three treatment groups show a significant lower mass than the control groups with the exception of the C57 combination group. The LDLr animals also show a profound hypercholesterolemia, significantly aided by the interventions, but not

bringing the levels back to a control state. The control animals also show significant cholesterol reductions within the treatment groups. Interestingly, the LDLr animals had a significantly larger outer diameter at the start of the experiments; however the active tone was significantly lower in all of the treatments groups of the control mice with no change in the LDLr group.

Figure 1 presents dilator reactivity for isolated arterioles from C57 and LDLr control mice in response to hypoxia (Panel A), and increasing concentrations of acetylcholine (Panel B), detaNONOate (Panel C), U-46619, a thromboxane mimetic (Panel D). In response to the endothelium-dependent dilation challenges of hypoxia and acetylcholine, responses in vessels from LDLr mice were attenuated compared to the C57 control responses. When L-NAME is added, inhibiting NOS activity, the LDLr animals show a mild attenuation of the dilator reactivity, while the control animals show a blunted response. In response to indomethacin, a cyclooxygenase inhibitor, there is a mild reduction in control activity, while the LDLr group shows a greater loss of reactivity. In combination, these two inhibitors lead to an abolishment of control activity, however, there is a significant endothelium-dependent arteriolar dilation remaining in the LDLr animals. In Panel C (detaNONOate), where dilator responses are endothelium-independent, or in Panel D (U-46619), there were no significant differences at control or with the inclusion of either of the inhibitors, singly or in combination.

Figure 2 presents vascular reactivity of isolated skeletal muscle resistance arterioles in control and after cholesterol lowering treatment at control and following pharmaceutical inhibition of eNOS (L-NAME) and cyclooxygenase (INDO), and the combination of both pathways in response to hypoxia (Panel A) and to increasing concentrations of acetylcholine (Panel B), DetaNONOate (Panel C), and U-46619 (Panel D), of C57 mice, while Figure 3 presents these outcomes in LDLr mice. In response to the endothelium-dependent dilation challenges of hypoxia and acetylcholine C57 mice demonstrated a decreased arteriolar reactivity after pharmaceutical therapy; however these changes are not mimicked within the LDLr mice.

When challenged with L-NAME (Panel B) this significant difference between the control and pharmaceutically treated control is evident, as there is a residual dilation associated with the cholesterol lowering treatment. There are no significant differences between the groups with the COX inhibitor indomethacin (Panel B) or in combination with NOS inhibition (Panel B). There were no significant differences using detaNONOate (Panel C) or U-46619 (Panel D), with respect to the pharmaceutical treatment in either the control or hypercholesterolemic group.

Figure 4 presents control vascular reactivity and following pharmaceutical inhibition of eNOS (L-NAME) and cyclooxygenase (INDO), and the combination of both pathways in response to hypoxia (Panel A) and to increasing concentrations of acetylcholine (Panel B), DetaNONOate (Panel C), and U-46619 (Panel D) of isolated arterioles of C57 and LDLr mice (Figure 5) under control conditions and after exercise treatment. Hypoxia-induced dilation was pronounced in vessels that were within the exercise treatment from C57 and demonstrated a progressive attenuation as L-NAME and Indo were added to the bath, individually and in combination. Additionally, these significant differences were mirrored within the acetylcholine treatment. There were no significant differences within the control group with respect to the increasing concentrations of DetaNONOate (Panel C) or U-46619 (Panel D). There were no significant differences within the LDLr groups.

Figure 6 presents vascular reactivity of control and cholesterol lowering therapies combined with exercise in response to hypoxia (Panel A) and to increasing concentrations of acetylcholine (Panel B), DetaNONOate (Panel C), and U-46619 (Panel D) in isolated arterioles of C57 and LDLr (Figure 7) mice under control conditions and following pharmaceutical inhibition of eNOS (L-NAME) and cyclooxygenase (INDO), and the combination of both pathways. At control, both the C57 and LDLr animals show an attenuation of activity with respect to the combination treated counterparts after hypoxia and increasing concentrations of acetylcholine at baseline. With respect to the inhibitors, the C57 group shows differences

with the combination L-NAME/Indo inhibition; whereas LDLr animals show significance between with all inhibitors, L-NAME, Indo, and the combination of the two. There were no significant differences when challenged to DetaNONOate (Panel C) or U-46619 (Panel D).

Figure 8 presents the representative western blot and densitometry analysis of eNOS. These figures indicate that there are no significant differences between the groups.

Lastly, Table 2 presents the cytokine and chemokine analysis. This figure shows significantly higher values of citrate synthase within the exercise and combination treatments compared to the control and pharmaceutical treatment groups; however the combination group was also significantly lower when compared to the exercise group alone. The LDLr exercise group, with respect to nitrotyrosine is significantly lower than all other groups, including the control and the paired C57 group. With respect to CRP, the exercise groups are significantly lower than any of the treatment or control groups; additionally, the combination treatment groups are significantly lower than the control and pharmaceutical treatment groups, but not as effective as exercise alone. With respect to e-selectin, the control groups are significantly higher than all of the treatment groups. The MMP-9 results show that the combination treatment groups are significantly higher than the other groups. In regard to icam, the control exercise group is significantly higher than the other groups, while in the LDLr groups, the treatment groups are significantly different than the control; however the direction is inconsistent. However, with respect to vcam, the control and pharmaceutical groups are significantly higher than the exercise and combination treatments. When evaluating IL-6 and IL-10 levels, the LDLr control group is significantly higher than any of the treatment groups, while no significant differences are seen within the C57 groups.

DISCUSSION

While the evolution of hypercholesterolemia has been shown to be a key risk factor leading to the development of cardiovascular disease, due to modifications to vascular structure and endothelial

function, alterations within the microcirculation is directly coupled to endothelial cell dysfunction (3; 8; 15; 18; 39; 49). Previous studies have shown this dysfunction to be directly related to decreases in vascular nitric oxide (NO) bioavailability, elevated oxidant stress, and a strongly pro-inflammatory state; however these prior studies have not investigated interventions which may improve these consequences and, in turn, decrease cardiovascular disease risk (3; 4; 12; 19; 31; 39; 41; 42; 46; 48).

The primary observation of this study is that the traditional interventional strategies of pharmaceutical therapy and exercise treatment associated with hypercholesterolemia are not effective strategies to improve vascular reactivity (Figure 2-4) or increase eNOS expression (Figure 8); however, exercise alone was shown to be effective to improve oxidative stress and inflammation (Table 2). Unexpectedly, the pharmaceutical and combination therapies were shown to be detrimental to the control group (Figure 2 and 4). However, the mechanism of the decrease is not directly related to changes associated with oxidative stress or inflammation (Table 2). This decrease may be due to asymmetric dimethylarginine (ADMA) an endogenous inhibitor of eNOS activity due to competition with L-arginine leading to a reduction of nitric oxide bioavailability (22). ADMA has been shown to be elevated in hypercholesterolemic mice and show strong correlations to a decrease in low-mediated dilation in human hypercholesterolemic populations (2; 46; 47).

Previous studies have suggested that the condition of hypercholesterolemia leads to a decrease in NO bioavailability and therefore compensatory mechanisms of arachidonic acid metabolism act to maintain endothelium-dependent dilation (12; 39). Additional studies have shown that this decrease in NO bioavailability may be due to a pro-inflammatory state coupled with high oxidative stress, leading to oxidative scavenge of available NO compounded with eNOS uncoupling, creating the perfect storm to decrease NO (42; 43).

The rescue of NO has been shown to be linked to a number of pleiotropic effects of pharmaceutical treatments currently available on the market. These drugs have been shown to reduce inflammation and oxidative stress, despite elevated cholesterol levels. Some studies have shown positive results with respect to lipid peroxidation, including the increase of an antioxidant effect leading to a decrease in ox-LDL with combination ezetimibe/statin treatment (17). A number of studies are currently ongoing to determine if the anti-inflammatory and anti-oxidative pleiotropic effects would be beneficial to normocholesterolemic patients, specifically to managing inflammation and preventing atherosclerotic lesions (44; 51). These studies have been met with mixed reviews and results. Many of these results have been difficult to discern in traditional studies, as discrete results in the hypercholesterolemic populations are very similar to results directly attributed to the cholesterol lowering benefit. Our results suggest that these pharmaceutical agents may in fact be detrimental to patients who are not hypercholesterolemic.

It has previously been demonstrated that a moderate intensity exercise program can also lead to an increase in NO bioavailability, through acute shear stresses, improvements in mitochondrial anti-oxidative mechanisms, and through a decrease in the low-grade pro-inflammatory phenotype due to myokine activation (7; 26; 29; 49). CRP has been shown to reduce eNOS activity, promote eNOS uncoupling, and alter eNOS phosphorylation, overall limiting NO bioavailability (33; 35; 45). In this study, while exercise did not lead to a significant improvement in vascular reactivity, improvements to overall oxidative stress and inflammatory marker profile were evident with respect to CRP, MCP-1, RANTES, and nitrotyrosine. This suggests that overall improvements are being made to the mechanisms affecting NO bioavailability, and perhaps improvements to reactivity may be evident over a longer training period.

Combining the two therapies used in this study lead to a combination of the results seen independently. Interestingly, the combination treatment leads to a detriment to the vascular reactivity, in both the control and hypercholesterolemic groups. While only a moderate improvement was made with respect to CRP and RANTES levels, unlike the drastic improvements seen with exercise alone. In icam and vcam measurements, significant reductions were also evident when comparing the combination groups to the control and single interventions. However, this improvement was not evident when examining nitrotyrosine levels. Interestingly, the combination treatment seems to impact to amount of or efficiency of the exercise that is performed, as citrate synthase levels are significantly lower than that of the exercise groups independently. However, at present, the side effects of the combined therapy are not well described, and it is unclear how effective these are for impacting the inflammatory and oxidative profiles (21; 23; 36).

Taken together, the results of the present study suggest that exercise alone may be the most beneficial intervention for hypercholesterolemic condition. However, as our model only evaluated the pleiotropic effects of the ameliorative therapies, with respect to human patient, the exercise treatment in combination with the direct cholesterol lowering effects of pharmaceutical treatment, may lead to the greatest benefit. However, this treatment is not suggested to a population who are considered normocholesterolemic, as the mechanisms leading to the detriment to the vascular reactivity within this group need further exploration. Additionally, the anti-oxidative and anti-inflammatory benefits pertaining to the exercise only group are much greater than the pleiotropic effects within the pharmaceutical or combination groups. In conclusion, the mechanisms specific to improvements within the microcirculation of hypercholesterolemic patients warrants further review. The interventional strategies need to be evaluated with respect to their direct cholesterol lowering benefits and pleiotropic effects within a normocholesterolemic and hypercholesterolemic populations.

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FIGURE AND TABLE LEGENDS

Table 1. Baseline and treatment characteristics of mice and individual arterioles used in the present study. *p<0.05 vs. C57.

	Control C57	**Control LDLr**	**Statin C57**	**Statin LDLr**	**Exercise C57**	**Exercise LDLr**	**Combo C57**	**Combo LDLr**
Age (weeks)	21.78 ± 0.18	21.00† ± 0.15	21.33 ± 0.31	22.14* ± 0.33	22.29 ± 0.07	22.17* ± 0.13	24.50 ± 0.14	24.67* ± 0.09
Weight	32.14 ± 0.28	31.77† ± 0.37	27.47 ± 0.26	28.19* ± 0.29	26.43 ± 0.20	26.43* ± 0.58	26.73 ± 0.15	24.30* ± 0.08
MAP	87.00 ± 1.27	87.20† ± 1.18	78.00* ± 1.17	77.57*† ± 1.12	80.29* ± 1.38	71.33*† ± 1.55	87.00 ± 2.54	77.60*† ± 1.79
Weeks Pharmaceuticals /Exercise	-	-	9.35 ± 0.31	9.98 ± 0.35	4.71 ± 0.07	4.76 ± 0.08	13.79 ± 0.11	13.67 ± 0.08
							5.00 ± 0.10	4.90 ± 0.07
Passive Diameter								
Inner Pre	44.67 ± 1.23	46.50 ± 1.30	50.33 ± 2.34	44.86 ± 0.89	51.43 ± 1.41	55.50 ± 2.62	50.83 ± 1.63	46.17 ± 1.61
Outer Pre	69.22 ± 1.25	73.33† ± 1.74	69.50 ± 2.83	66.00† ± 0.93	68.14 ± 1.50	70.83† ± 2.61	69.33* ± 1.51	65.33† ± 2.30
Inner Post	70.89 ± 1.05	68.83† ± 1.60	71.50 ± 2.23	67.14*† ± 1.27	77.71 ± 1.35	85.67 ± 2.32	76.17 ± 1.70	71.17* ± 1.45
Outer Post	90.89 ± 1.03	85.17 ± 2.64	88.83 ± 2.77	83.14 ± 1.05	88.14 ± 1.52	96.50 ± 2.06	89.67* ± 2.00	84.67 ± 1.78
Active Tone (%)	37.89 ± 1.17	33.53† ± 0.83	31.26* ± 1.29	32.99 ± 0.81	34.07* ± 1.19	36.10 ± 1.82	33.98* ± 1.11	35.66 ± 1.64
Total Cholesterol	103.30 ± 2.48 (n=8)	236.84† ± 2.92 (n=10)	79.16* ± 1.26 (n=9)	199.05*† ± 5.10 (n=9)	67.74* ± 0.99 (n=10)	213.03*† ± 3.18 (n=10)	83.89* ± 4.21 (n=10)	184.92*† ± 3.29 (n=10)

Table 2. Baseline and treatment plasma profile characteristics of mice used in the present study. * $p<0.05$ vs. C57.

	Control C57	Control LDLr	Statin C57	Statin LDLr	Exercise C57	Exercise LDLr	Combo C57	Combo LDLr
CRP (ng/mL)	82.93 ± 3.46	81.12 ± 2.58	78.66 ± 1.47	79.73 ± 1.05	33.52 * ± 0.61	35.52 * ± 0.30	50.55 * ± 0.85	52.79 * ± 1.05
IL-1B (pg/mL)	46.69 ± 7.09	27.12 ± 1.01	22.40 ± 2.04	31.25 ±4.59	3.22 ± 0.00	174.03 *† ± 94.97	35.86 ± 4.46	21.57 ± 0.69
IL-6 (pg/mL)	54.61 ± 6.80	70.44 ± 16.09	27.81 ± 4.57	11.99 ± 0.60	n/a	5.82 ± 1.95	44.86 ± 12.23	14.51 ± 0.63
IL-10 (pg/mL)	50.02 ± 4.72	142.20 † ± 33.70	36.94 ± 2.56	23.65 * ± 1.57	n/a	64.35 ±0.00	23.15 ± 3.85	14.48 † ± 0.73
e-selectin	132.23 ± 12.04	88.99 † ± 5.49	78.43 * ± 9.03	51.95 *† ± 1.60	41.08 * ± 0.56	53.84 * ±1.21	46.09 * ± 1.17	45.58 * ± 1.76
mmp-9	2.27 ± 0.21	2.98 ± 0.24	1.73 ± 0.11	1.35 ±0.12	3.09 * ± 0.38	2.11 *† ± 0.13	6.00 * ± 0.23	4.39 *† ±0.17
icam	1098.34 ± 14.04	984.80 † ± 23.26	1169.47 ± 24.05	1067.76 * ± 18.35	1226.20 * ± 27.39	1090.05 *† ± 29.03	956.57 * ± 31.54	872.91 *† ± 38.03
Vcam	20.77 ± 0.49	18.30 *± 0.35	18.34 *± 0.51	17.45 ± 0.32	14.87 * ± 0.46	15.20 * ± 0.41	14.48 * ± 0.27	13.91 * ± 0.36
Nitrotyrosine	3.03 ± 0.10	3.59 ± 0.19	2.14 ± 0.08	3.16 ± 0.63	2.83 ± 0.24	1.91 *†± 0.25	3.05 ± 0.18	3.22 ± 0.20
TNFα	4.34 ± 0.28	2.90 ± 0.30	4.08 ± 0.45	2.23 ± 0.00	3.09 * ± 0.23	5.23 *± 0.12	2.61 *± 0.17	4.44 * ± 0.24
Citrate Synthase	51.06 ± 4.82	53.47 ± 4.17	59.32 ± 2.39	63.17 ± 2.96	155.12 * ± 13.35	173.99 * ± 19.65	104.16 * ± 6.62	90.62 * ± 6.57

Figure 1. Data describing the dilator reactivity of isolated skeletal muscle resistance arterioles under control conditions of C57 and LDLr mice and following pharmacological inhibition of nitric oxide synthase with L-NAME, cyclooxygenase with indothemation (INDO) or combined inhibition of both enzymatic pathways in response to hypoxia (Panel A), and increasing concentrations of acetylcholine (Panel B), detaNONate (Panel C) and describing the constrictor reactivity in response to increasing concentrations of U-46619 (Panel D). Data, presented as mean±SEM, are shown for arterioles under control conditions and associated stimuli. n=4-14 animals for each strain; * $p<0.05$ vs. C57; † $p<0.05$ vs. control within that strain.

Figure 2. Data describing the dilator reactivity of isolated skeletal muscle resistance arterioles of C57 mice at control and pharmaceutically treated conditions at control and following pharmacological inhibition of nitric oxide synthase with L-NAME, cyclooxygenase with indothemation (INDO) or combined inhibition of both enzymatic pathways in response to hypoxia (Panel A) and increasing concentrations of acetylcholine (Panels B), detaNONate (Panel C) and constrictor reactivity to increasing concentrations of U-46619 (Panel D). n=4-14 animals for each strain; * $p<0.05$ vs. C57; † $p<0.05$ vs. control within that strain.

Figure 3. Data describing the dilator reactivity of isolated skeletal muscle resistance arterioles of LDLr mice at control and pharmaceutically treated conditions at control and following pharmacological inhibition of nitric oxide synthase with L-NAME, cyclooxygenase with indothemation (INDO) or combined inhibition of both enzymatic pathways in response to hypoxia (Panel A) and increasing concentrations of acetylcholine (Panels B), detaNONate (Panel C) and constrictor reactivity to increasing concentrations of U-46619 (Panel D). n=4-11 animals for each treatment; * $p<0.05$ vs. C57; † $p<0.05$ vs. control within that strain.

Figure 4. Data describing the dilator reactivity of isolated skeletal muscle resistance arterioles of C57 mice at control and exercised animals at control and following pharmacological inhibition of nitric oxide synthase with L-NAME, cyclooxygenase with indothemation (INDO) or combined inhibition of both enzymatic pathways in response to hypoxia (Panel A) and increasing concentrations of acetylcholine (Panels B), detaNONate (Panel C) and constrictor reactivity to increasing concentrations of U-46619 (Panel D). n=4-14 animals for each treatment; * $p<0.05$ vs. C57; † $p<0.05$ vs. control within that strain.

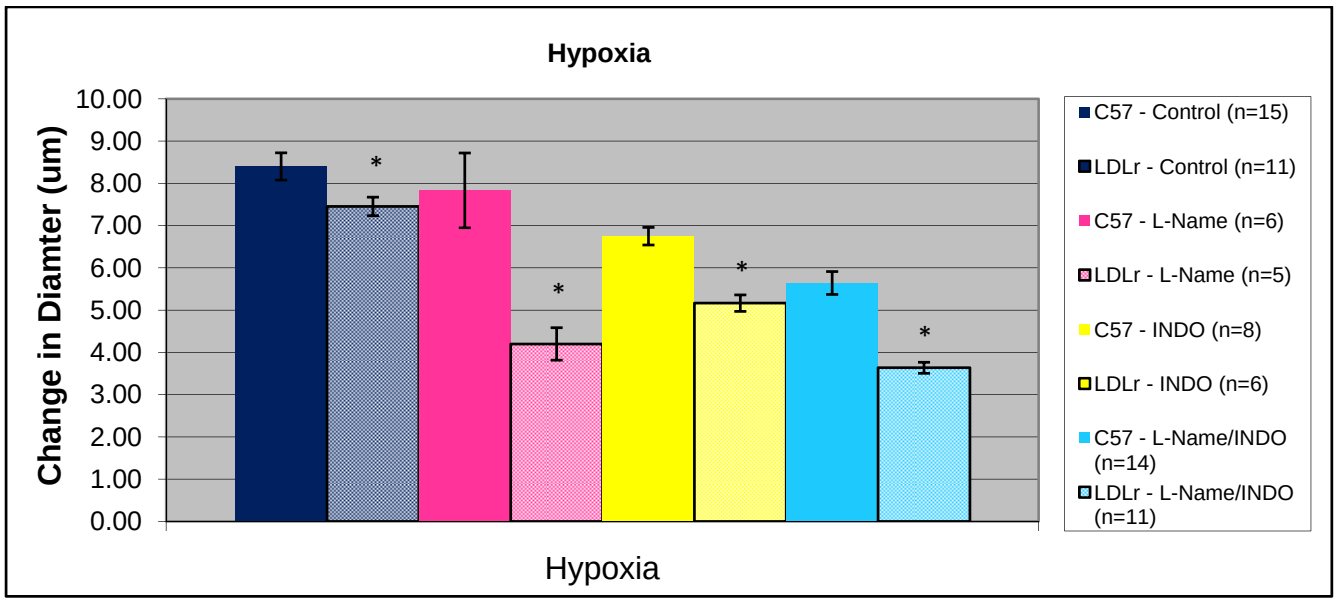
Figure 5. Data describing the dilator reactivity of isolated skeletal muscle resistance arterioles of LDLr mice at control and exercised animals at control and following pharmacological inhibition of nitric oxide synthase with L-NAME, cyclooxygenase with indothemation (INDO) or combined inhibition of both enzymatic pathways in response to hypoxia (Panel A) and increasing concentrations of acetylcholine (Panels B), detaNONate (Panel C) and constrictor reactivity to increasing concentrations of U-46619 (Panel D). n=4-11 animals for each treatment; * $p<0.05$ vs. C57; † $p<0.05$ vs. control within that strain.

Figure 6. Data describing the dilator reactivity of isolated skeletal muscle resistance arterioles of C57 mice at control and combination pharmaceutically treated and exercised animals at control and following pharmacological inhibition of nitric oxide synthase with L-NAME, cyclooxygenase with indothemation (INDO) or combined inhibition of both enzymatic pathways in response to hypoxia (Panel A) and increasing concentrations of acetylcholine (Panels B), detaNONate (Panel C) and constrictor reactivity to increasing concentrations of U-46619 (Panel D). n=4-14 animals for each treatment; * $p<0.05$ vs. C57; † $p<0.05$ vs. control within that strain.

Figure 7. Data describing the dilator reactivity of isolated skeletal muscle resistance arterioles of LDLr mice at control and combination pharmaceutically treated and exercised animals at control and following pharmacological inhibition of nitric oxide synthase with L-NAME, cyclooxygenase with indothemation (INDO) or combined inhibition of both enzymatic pathways in response to hypoxia (Panel A) and increasing concentrations of acetylcholine (Panels B), detaNONate (Panel C) and constrictor reactivity to increasing concentrations of U-46619 (Panel D). n=4-11 animals for each treatment; * $p<0.05$ vs. C57; † $p<0.05$ vs. control within that strain.

Figure 8. Representative western blot and densitometry data describing eNOS (Panel A) responses of pooled isolated skeletal muscle resistance arterioles of control C57 and LDLr mice in addition to pharmaceutically treated, exercised, and combination therapies mice. Data, presented as mean±SEM. n=3 animals for each group; * $p<0.05$ vs. control conditions in that strain.

A)


Hypoxia
Change in Diamter (um)
10.00
9.00
8.00
7.00
6.00
5.00
4.00
3.00
2.00
1.00
0.00
*
*
*
*
Hypoxia
C57 - Control (n=15)
LDLr - Control (n=11)
C57 - L-Name (n=6)
LDLr - L-Name (n=5)
C57 - INDO (n=8)
LDLr - INDO (n=6)
C57 - L-Name/INDO (n=14)
LDLr - L-Name/INDO (n=11)

B)

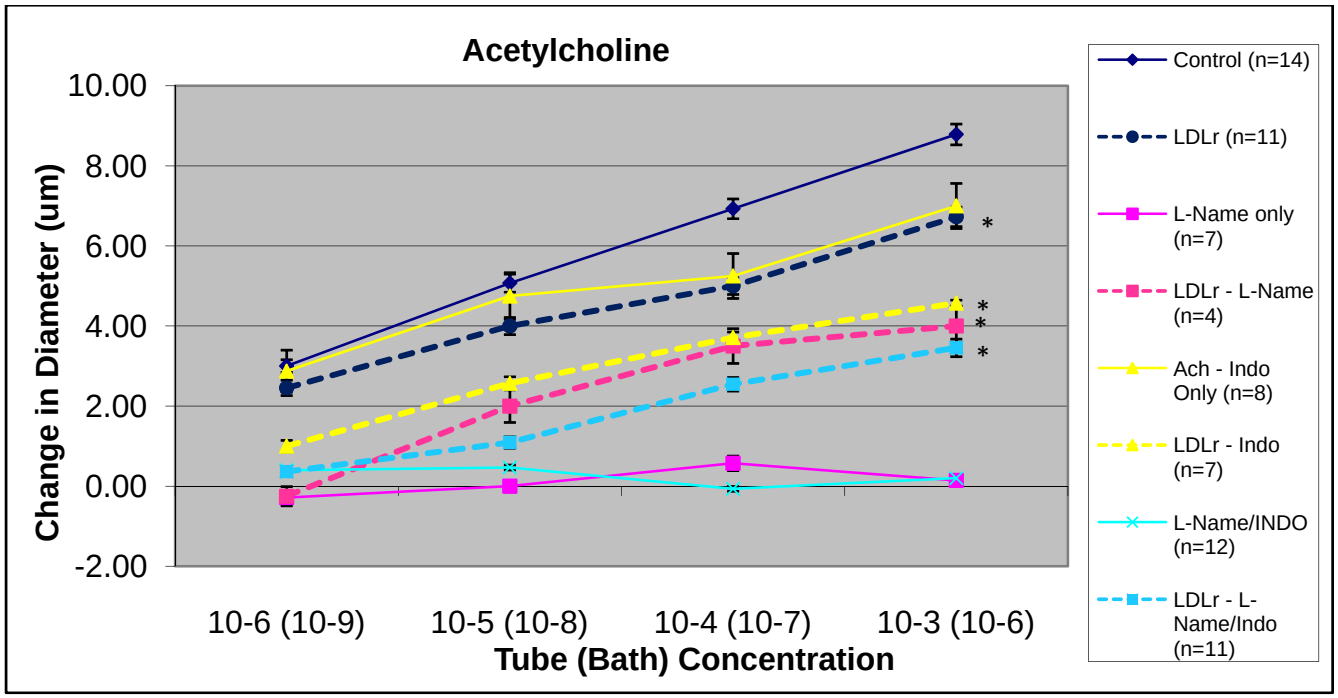
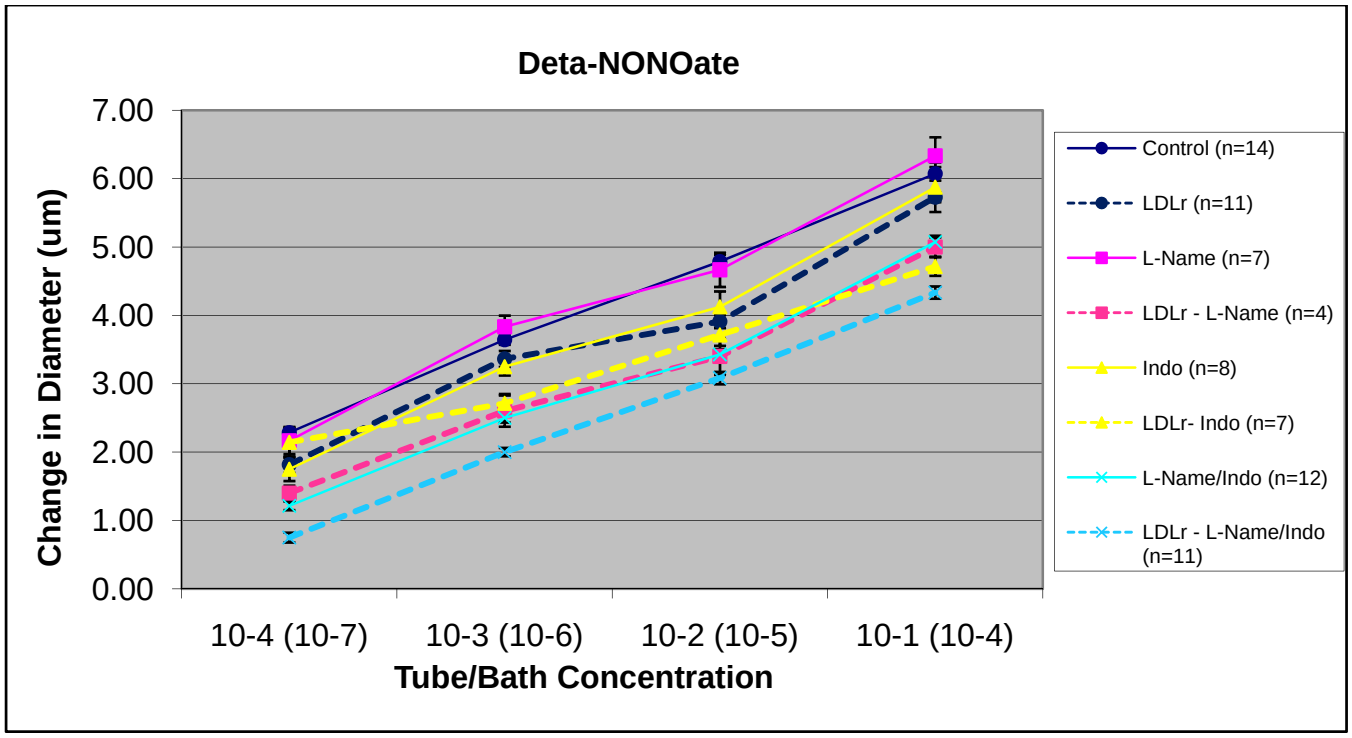

Acetylcholine
Change in Diameter (um)
10.00
8.00
6.00
4.00
2.00
0.00
-2.00
*
*
*
*
10-6 (10-9)
10-5 (10-8)
10-4 (10-7)
10-3 (10-6)
Tube (Bath) Concentration
Control (n=14)
LDLr (n=11)
L-Name only (n=7)
LDLr - L-Name (n=4)
Ach - Indo Only (n=8)
LDLr - Indo (n=7)
L-Name/INDO (n=12)
LDLr - L-Name/Indo (n=11)

Figure 1


Deta-NONOate
Change in Diameter (um)
7.00
6.00
5.00
4.00
3.00
2.00
1.00
0.00
10-4 (10-7)
10-3 (10-6)
10-2 (10-5)
10-1 (10-4)
Tube/Bath Concentration
Control (n=14)
LDLr (n=11)
L-Name (n=7)
LDLr - L-Name (n=4)
Indo (n=8)
LDLr- Indo (n=7)
L-Name/Indo (n=12)
LDLr - L-Name/Indo (n=11)

D)

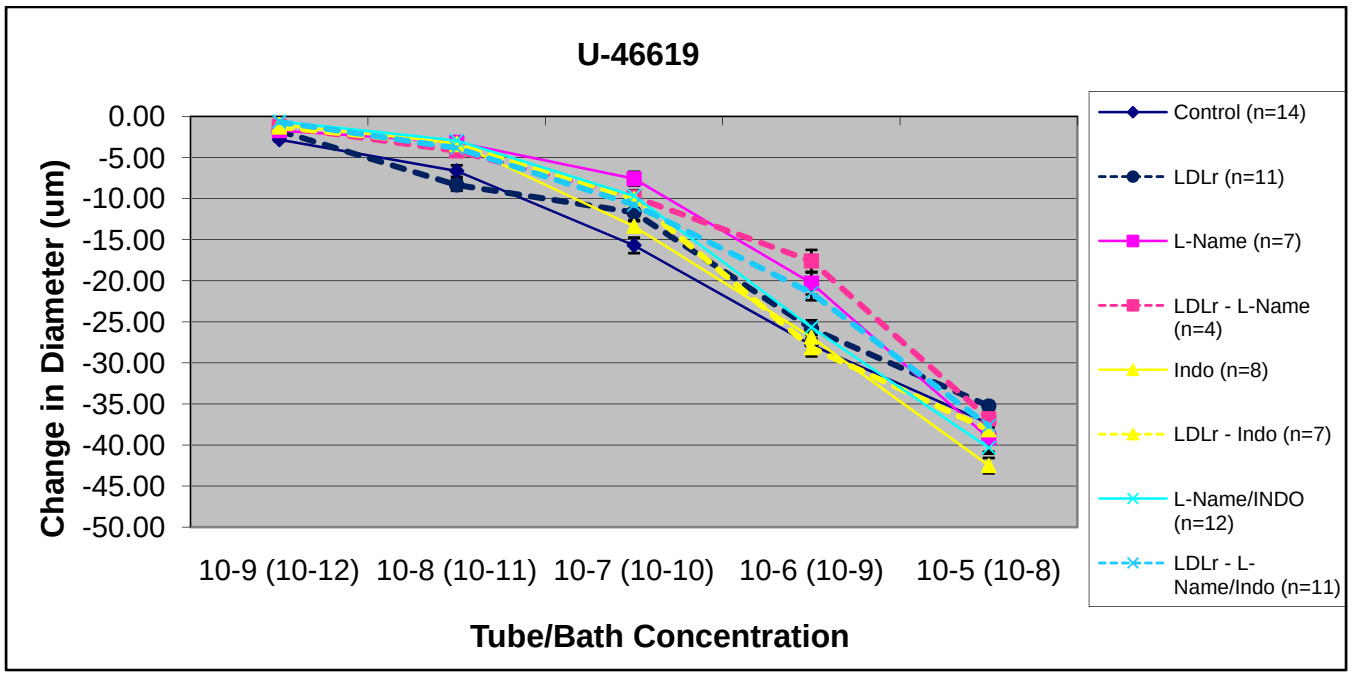
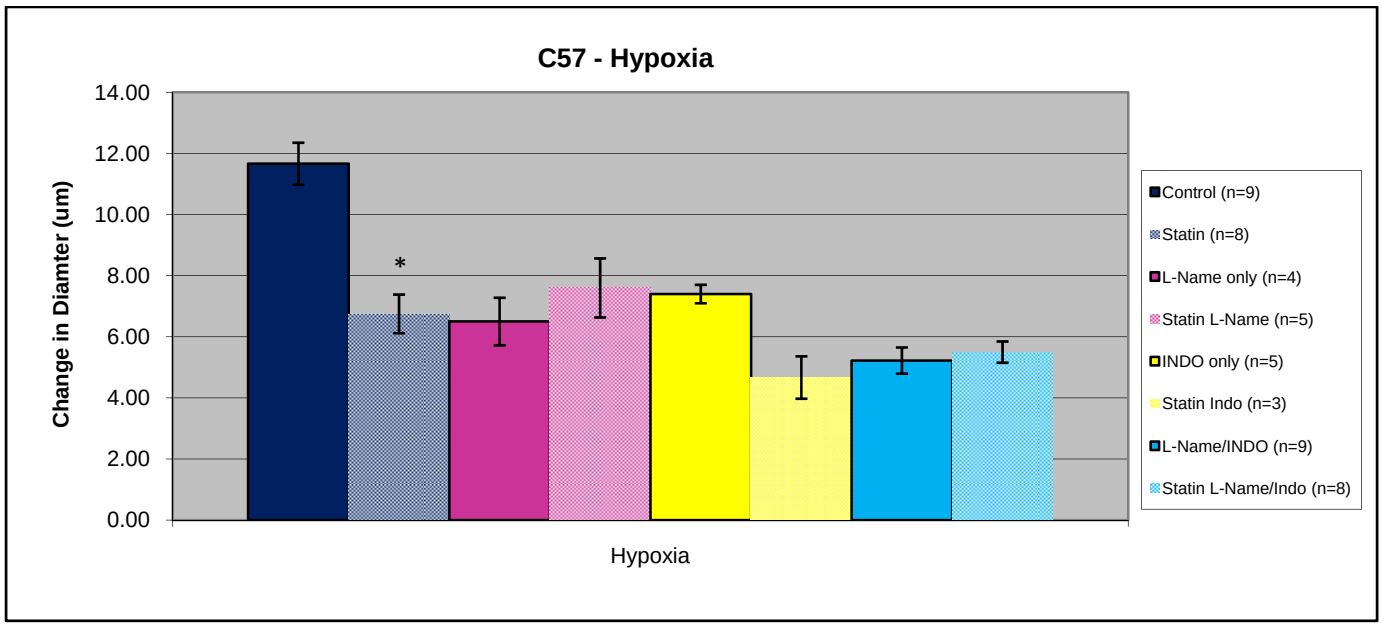

U-46619
Change in Diameter (um)
0.00
-5.00
-10.00
-15.00
-20.00
-25.00
-30.00
-35.00
-40.00
-45.00
-50.00
10-9 (10-12)
10-8 (10-11)
10-7 (10-10)
10-6 (10-9)
10-5 (10-8)
Tube/Bath Concentration
Control (n=14)
LDLr (n=11)
L-Name (n=7)
LDLr - L-Name (n=4)
Indo (n=8)
LDLr - Indo (n=7)
L-Name/INDO (n=12)
LDLr - L-Name/Indo (n=11)

Figure 1

A)


C57 - Hypoxia
Change in Diamter (um)
14.00
12.00
10.00
8.00
6.00
4.00
2.00
0.00
*
Hypoxia
Control (n=9)
Statin (n=8)
L-Name only (n=4)
Statin L-Name (n=5)
INDO only (n=5)
Statin Indo (n=3)
L-Name/INDO (n=9)
Statin L-Name/Indo (n=8)

B)

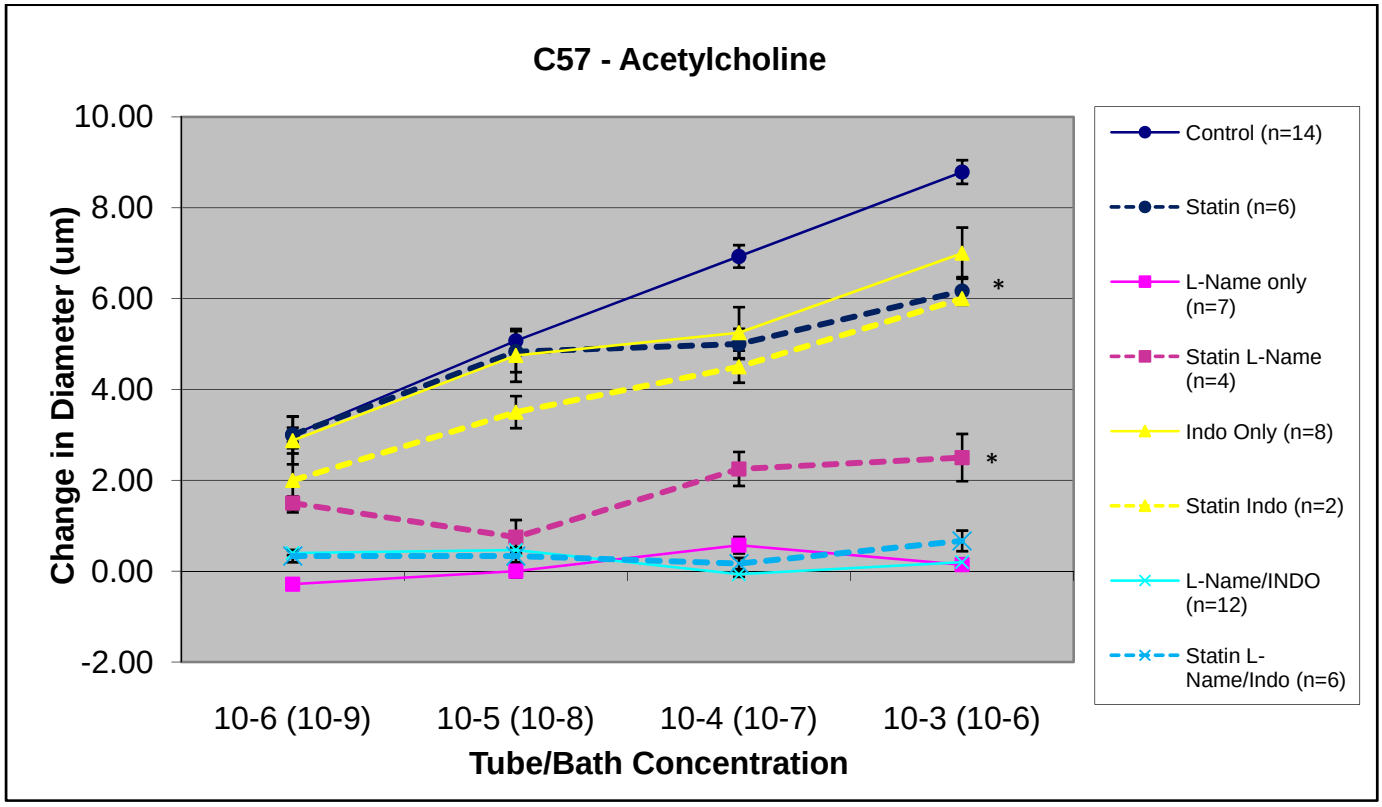
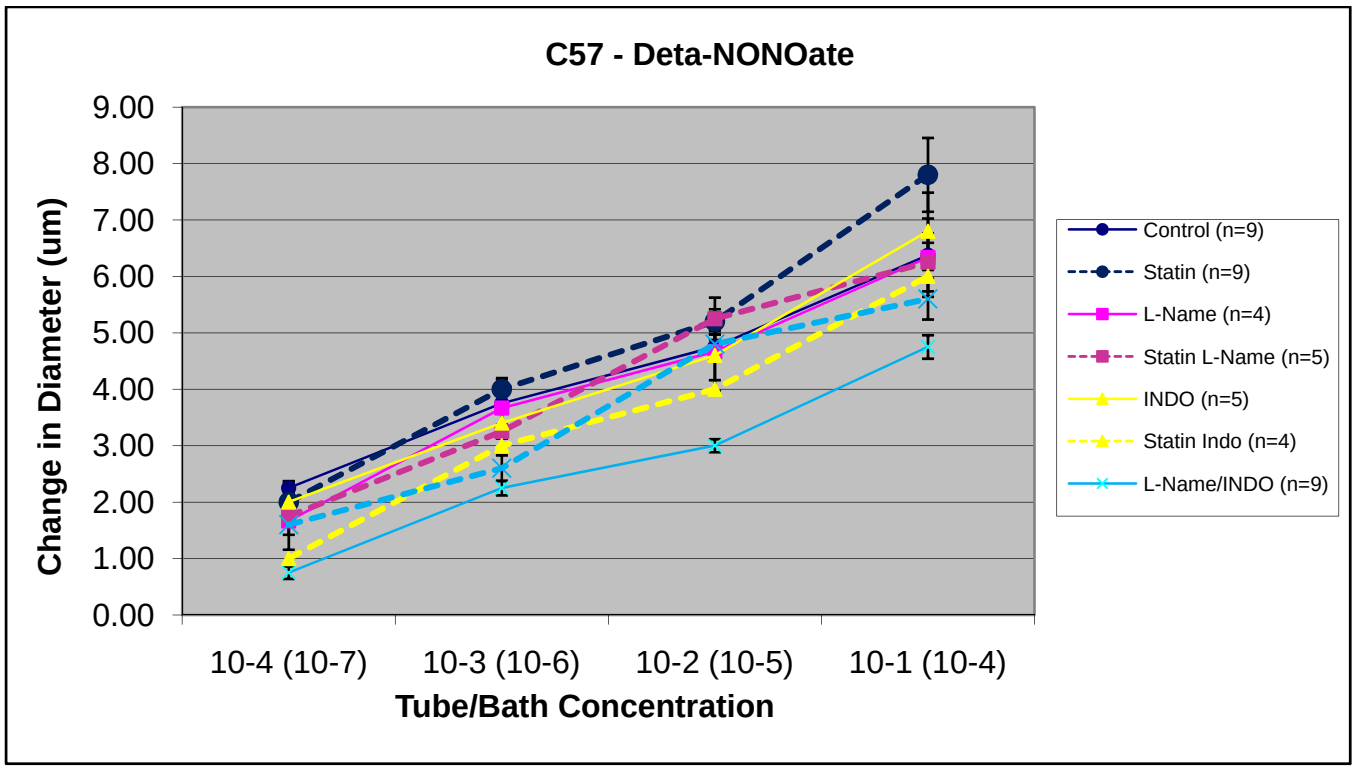

C57 - Acetylcholine
Change in Diameter (um)
10.00
8.00
6.00
4.00
2.00
0.00
-2.00
*
*
10-6 (10-9)
10-5 (10-8)
10-4 (10-7)
10-3 (10-6)
Tube/Bath Concentration
Control (n=14)
Statin (n=6)
L-Name only (n=7)
Statin L-Name (n=4)
Indo Only (n=8)
Statin Indo (n=2)
L-Name/INDO (n=12)
Statin L-Name/Indo (n=6)

Figure 2

C)


C57 - Deta-NONOate
Change in Diameter (um)
9.00
8.00
7.00
6.00
5.00
4.00
3.00
2.00
1.00
0.00
10-4 (10-7)
10-3 (10-6)
10-2 (10-5)
10-1 (10-4)
Tube/Bath Concentration
Control (n=9)
Statin (n=9)
L-Name (n=4)
Statin L-Name (n=5)
INDO (n=5)
Statin Indo (n=4)
L-Name/INDO (n=9)

D)

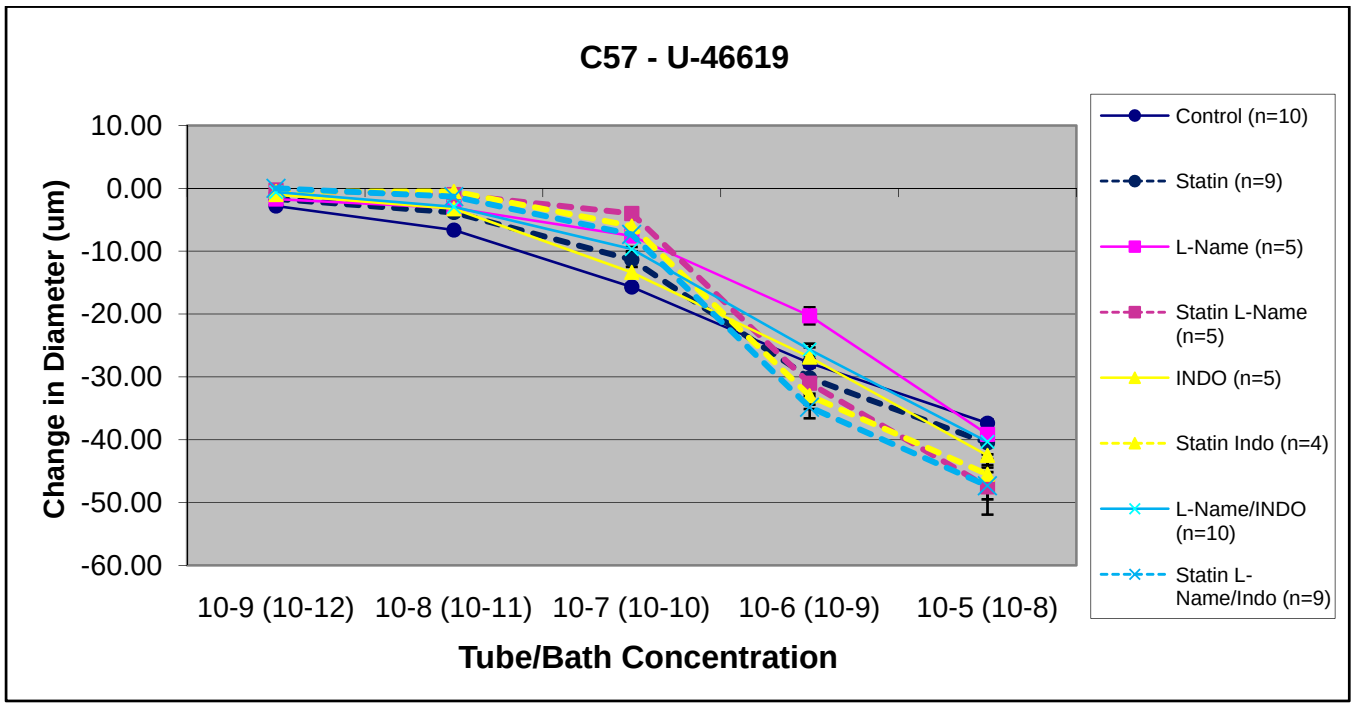
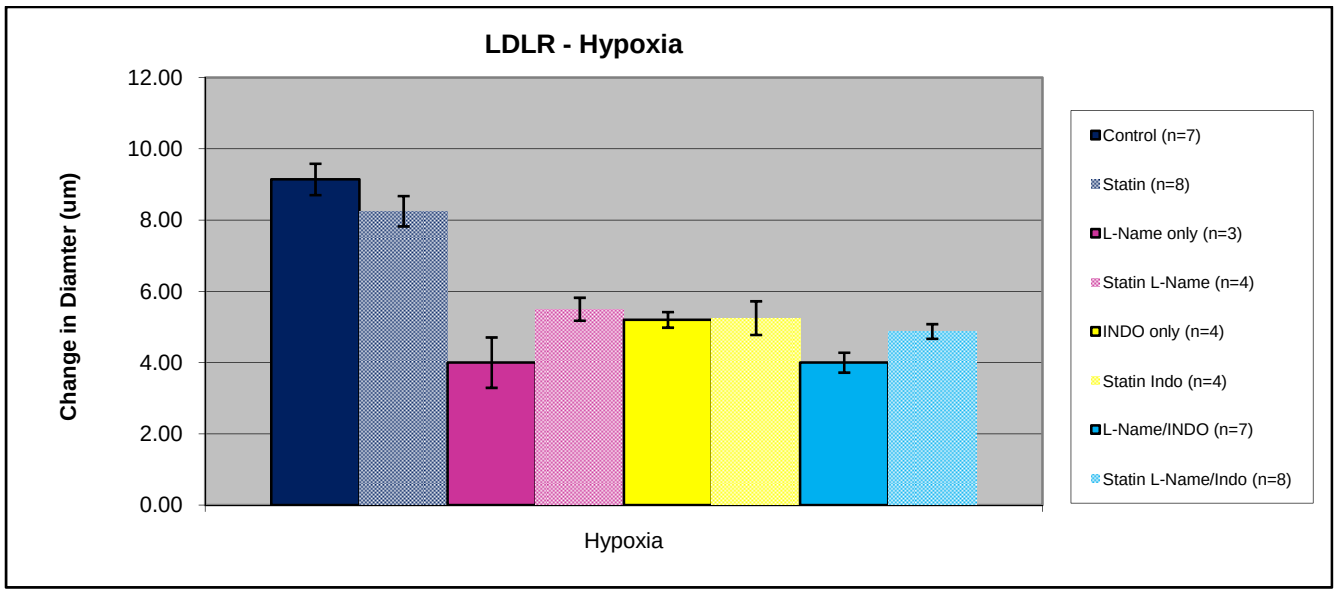

C57 - U-46619
Change in Diameter (um)
10.00
0.00
-10.00
-20.00
-30.00
-40.00
-50.00
-60.00
10-9 (10-12)
10-8 (10-11)
10-7 (10-10)
10-6 (10-9)
10-5 (10-8)
Tube/Bath Concentration
Control (n=10)
Statin (n=9)
L-Name (n=5)
Statin L-Name (n=5)
INDO (n=5)
Statin Indo (n=4)
L-Name/INDO (n=10)
Statin L-Name/Indo (n=9)

Figure 2

A)


LDLR - Hypoxia
Change in Diamter (um)
12.00
10.00
8.00
6.00
4.00
2.00
0.00
Hypoxia
Control (n=7)
Statin (n=8)
L-Name only (n=3)
Statin L-Name (n=4)
INDO only (n=4)
Statin Indo (n=4)
L-Name/INDO (n=7)
Statin L-Name/Indo (n=8)

B)

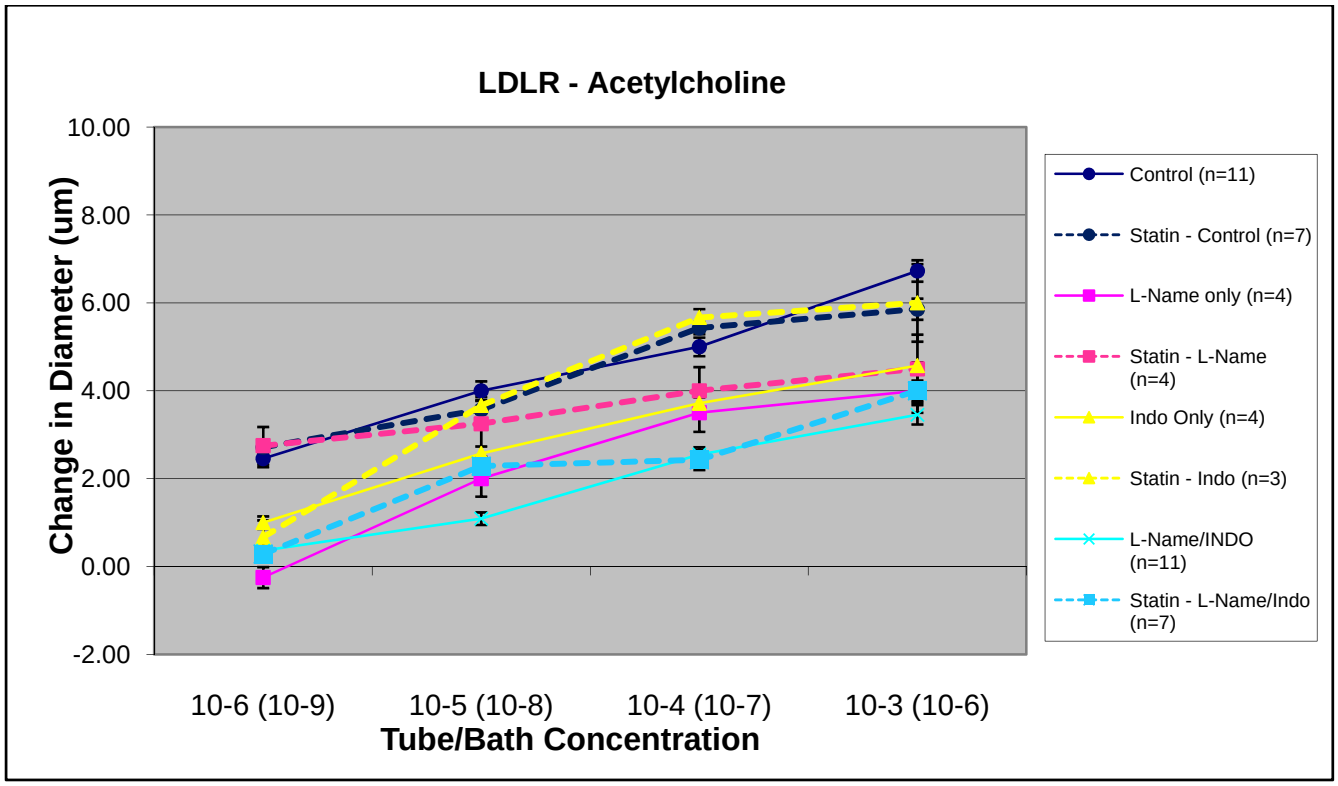
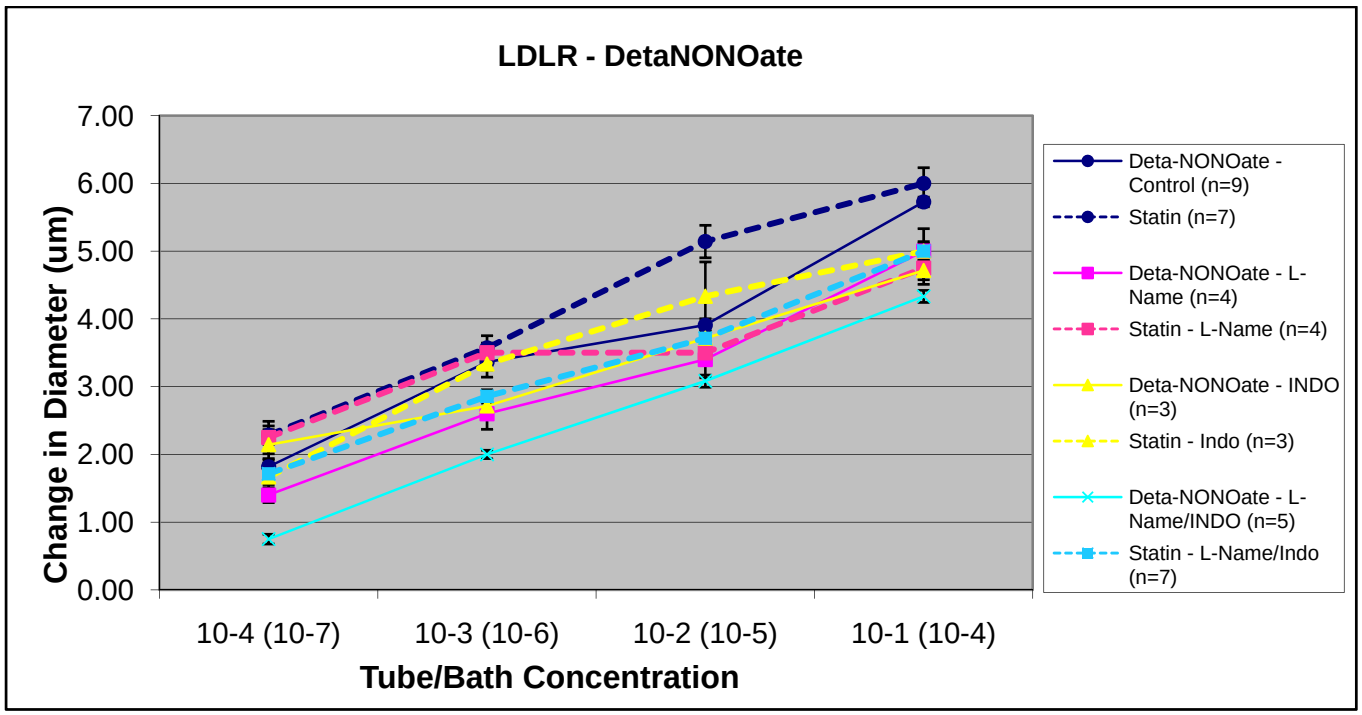

LDLR - Acetylcholine
Change in Diameter (um)
10.00
8.00
6.00
4.00
2.00
0.00
-2.00
10-6 (10-9)
10-5 (10-8)
10-4 (10-7)
10-3 (10-6)
Tube/Bath Concentration
Control (n=11)
Statin - Control (n=7)
L-Name only (n=4)
Statin - L-Name (n=4)
Indo Only (n=4)
Statin - Indo (n=3)
L-Name/INDO (n=11)
Statin - L-Name/Indo (n=7)

Figure 3

C)


LDLR - DetaNONOate
7.00
6.00
5.00
4.00
3.00
2.00
1.00
0.00
Change in Diameter (um)
10-4 (10-7)
10-3 (10-6)
10-2 (10-5)
10-1 (10-4)
Tube/Bath Concentration
Deta-NONOate - Control (n=9)
Statin (n=7)
Deta-NONOate - L-Name (n=4)
Statin - L-Name (n=4)
Deta-NONOate - INDO (n=3)
Statin - Indo (n=3)
Deta-NONOate - L-Name/INDO (n=5)
Statin - L-Name/Indo (n=7)

D)

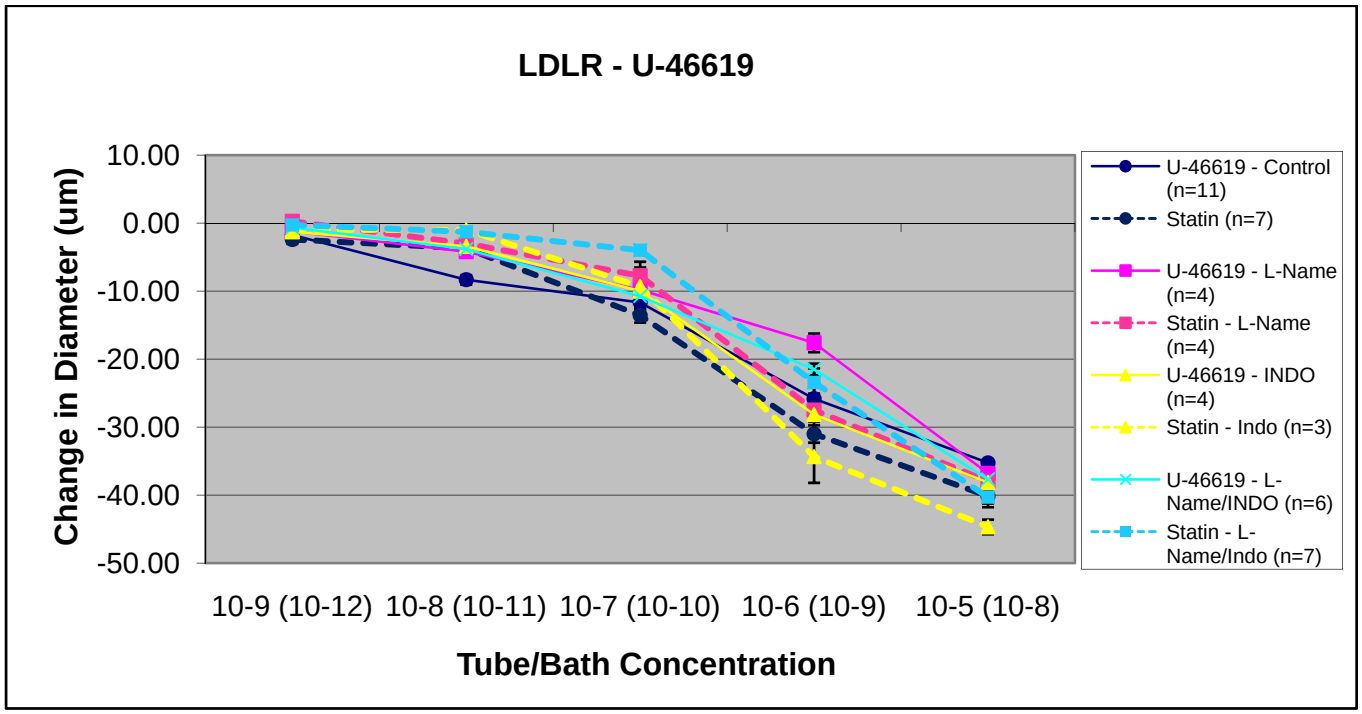
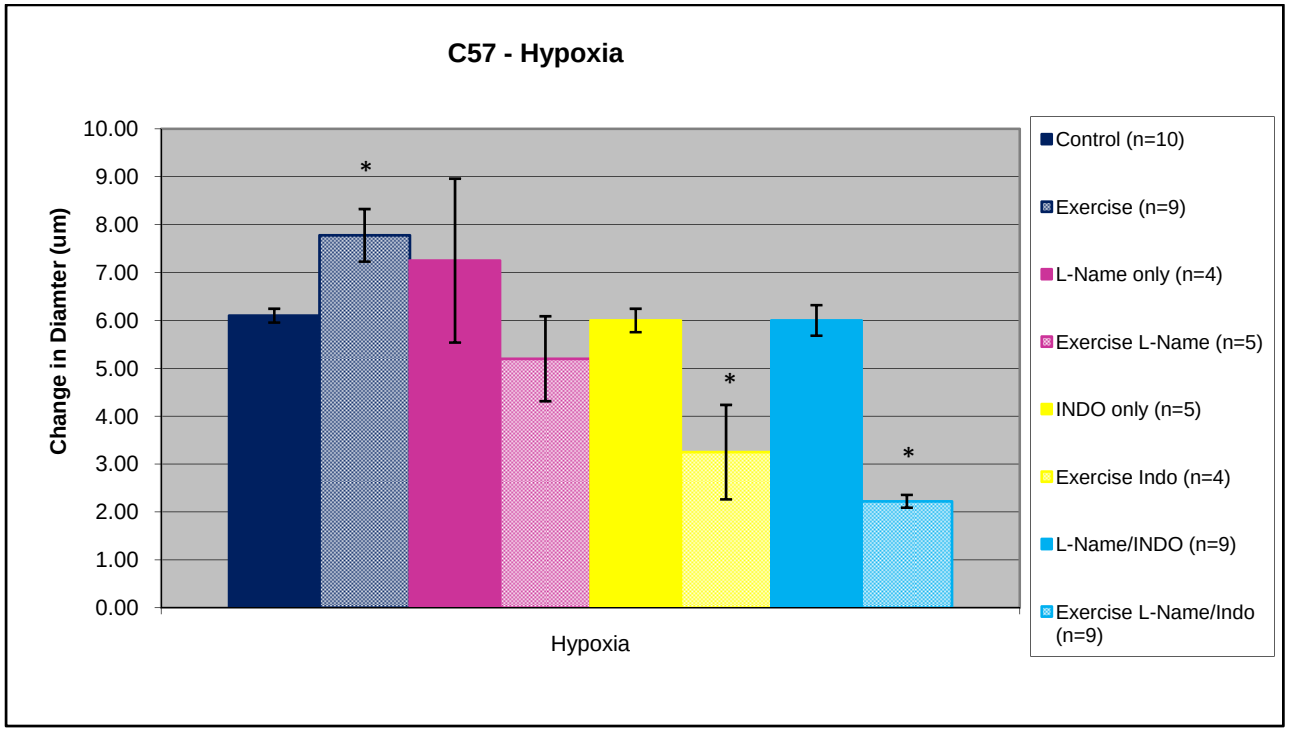

LDLR - U-46619
10.00
0.00
-10.00
-20.00
-30.00
-40.00
-50.00
Change in Diameter (um)
10-9 (10-12)
10-8 (10-11)
10-7 (10-10)
10-6 (10-9)
10-5 (10-8)
Tube/Bath Concentration
U-46619 - Control (n=11)
Statin (n=7)
U-46619 - L-Name (n=4)
Statin - L-Name (n=4)
U-46619 - INDO (n=4)
Statin - Indo (n=3)
U-46619 - L-Name/INDO (n=6)
Statin - L-Name/Indo (n=7)

Figure 3

A)


C57 - Hypoxia
Change in Diamter (um)
10.00
9.00
8.00
7.00
6.00
5.00
4.00
3.00
2.00
1.00
0.00
Hypoxia
Control (n=10)
Exercise (n=9)
L-Name only (n=4)
Exercise L-Name (n=5)
INDO only (n=5)
Exercise Indo (n=4)
L-Name/INDO (n=9)
Exercise L-Name/Indo (n=9)

B)

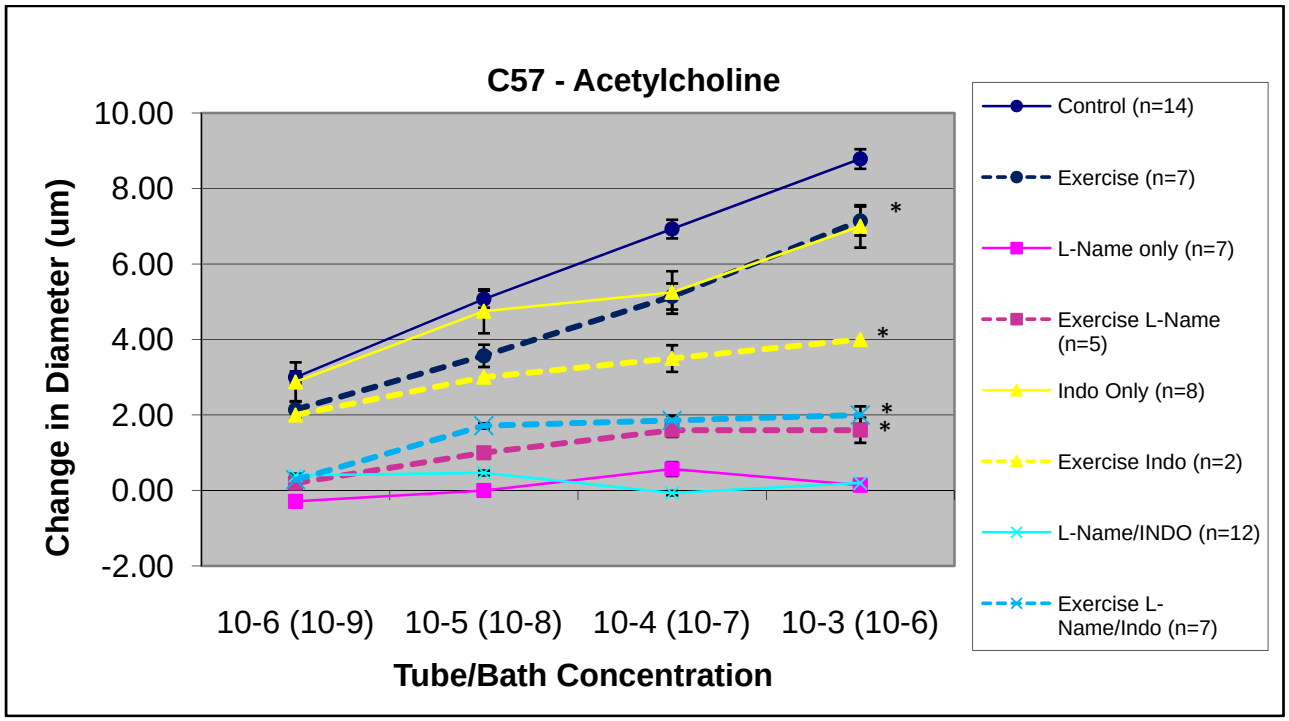
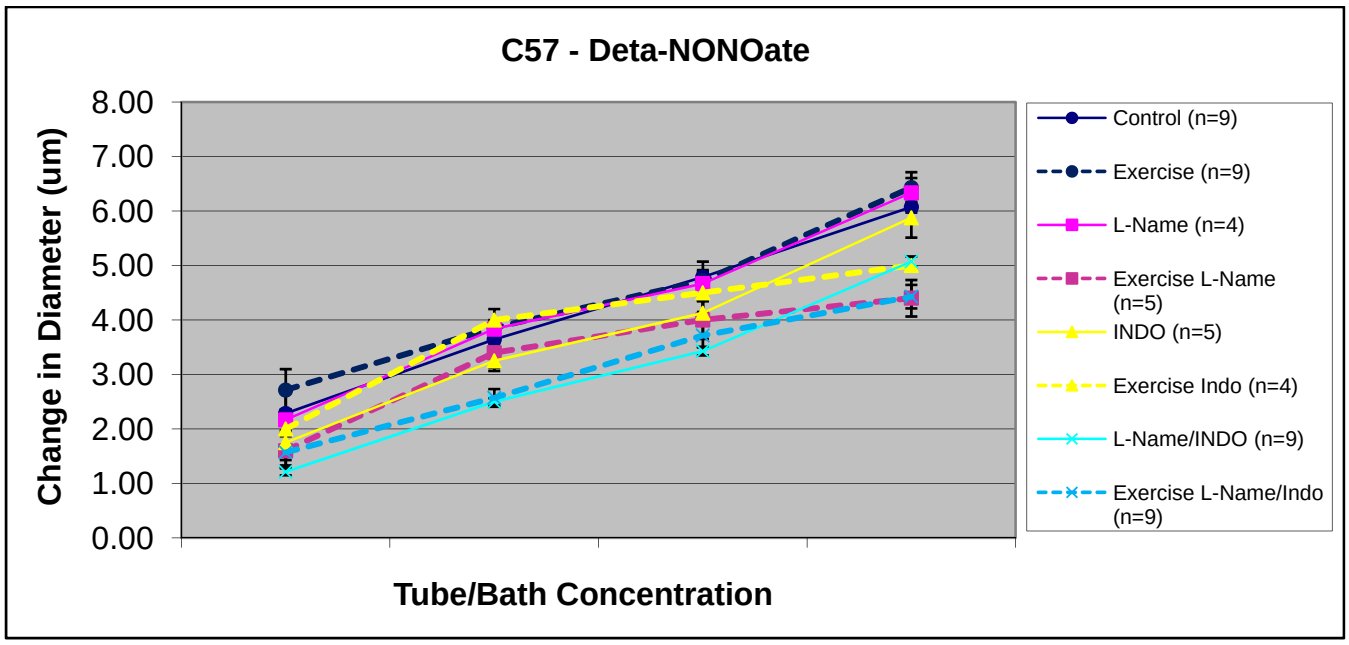

C57 - Acetylcholine
Change in Diameter (um)
10.00
8.00
6.00
4.00
2.00
0.00
-2.00
10-6 (10-9)
10-5 (10-8)
10-4 (10-7)
10-3 (10-6)
Tube/Bath Concentration
Control (n=14)
Exercise (n=7)
L-Name only (n=7)
Exercise L-Name (n=5)
Indo Only (n=8)
Exercise Indo (n=2)
L-Name/INDO (n=12)
Exercise L-Name/Indo (n=7)

Figure 4

C)


C57 - Deta-NONOate
Change in Diameter (um)
8.00
7.00
6.00
5.00
4.00
3.00
2.00
1.00
0.00
Tube/Bath Concentration
Control (n=9)
Exercise (n=9)
L-Name (n=4)
Exercise L-Name (n=5)
INDO (n=5)
Exercise Indo (n=4)
L-Name/INDO (n=9)
Exercise L-Name/Indo (n=9)

D)

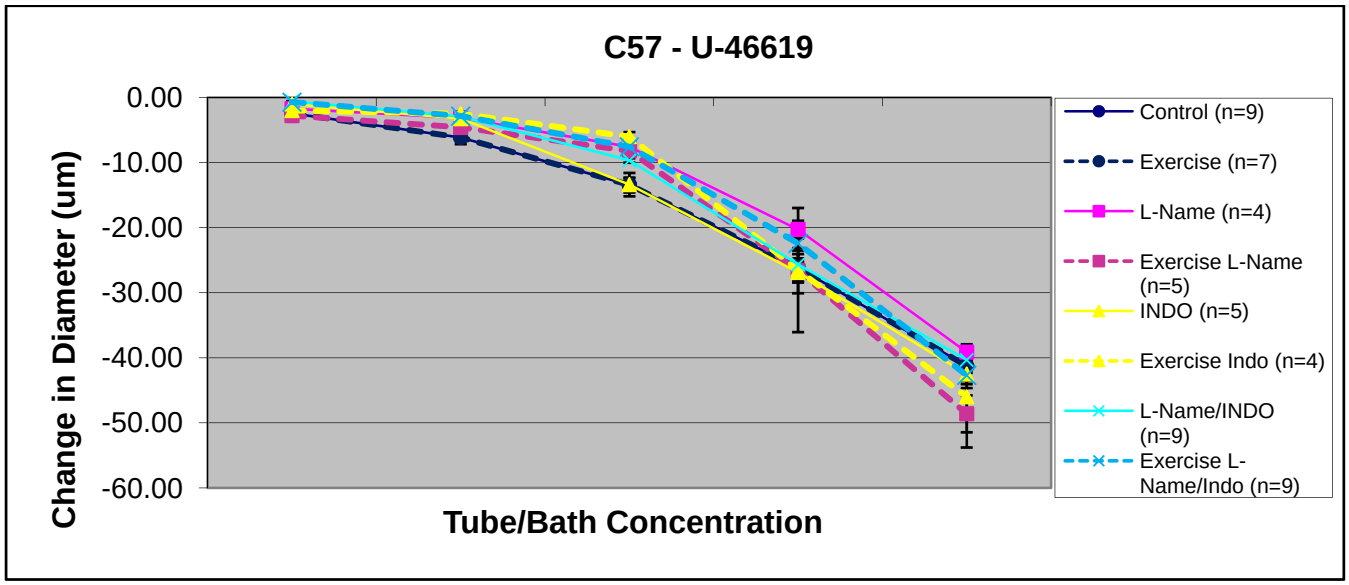
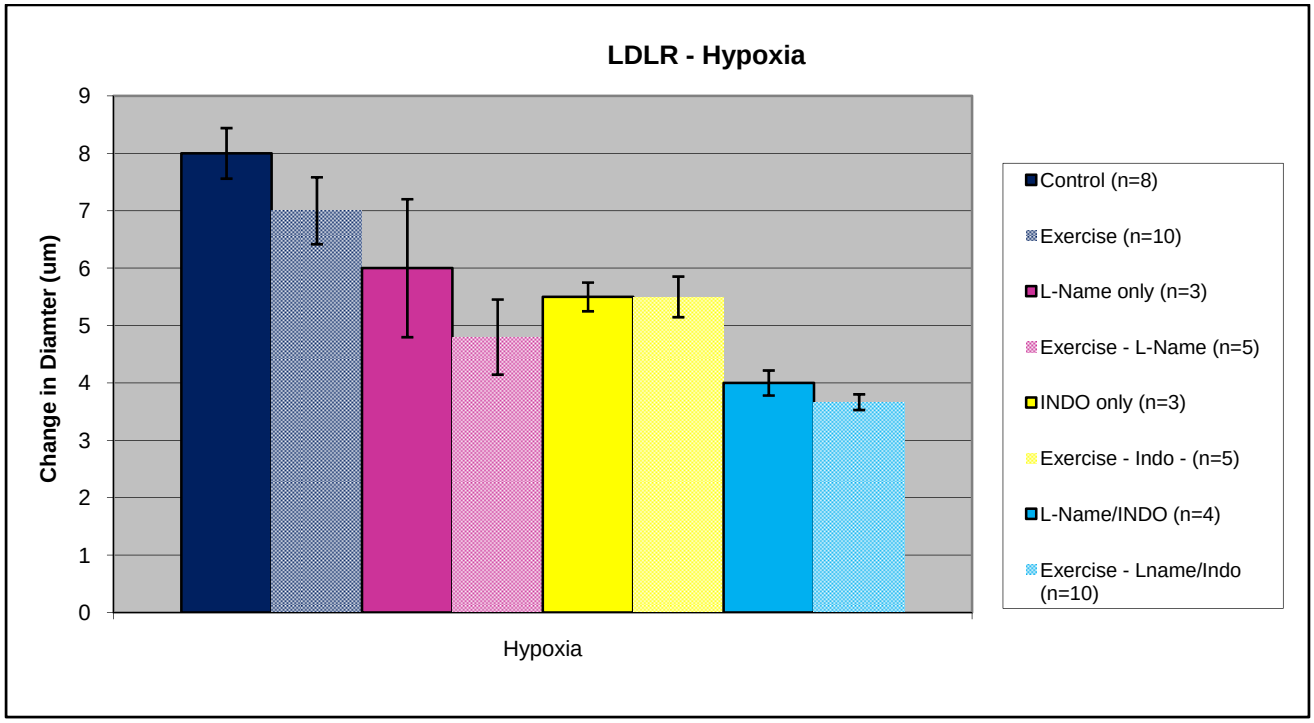

C57 - U-46619
Change in Diameter (um)
0.00
-10.00
-20.00
-30.00
-40.00
-50.00
-60.00
Tube/Bath Concentration
Control (n=9)
Exercise (n=7)
L-Name (n=4)
Exercise L-Name (n=5)
INDO (n=5)
Exercise Indo (n=4)
L-Name/INDO (n=9)
Exercise L-Name/Indo (n=9)

Figure 4

A)


LDLR - Hypoxia
Change in Diamter (um)
9
8
7
6
5
4
3
2
1
0
Hypoxia
Control (n=8)
Exercise (n=10)
L-Name only (n=3)
Exercise - L-Name (n=5)
INDO only (n=3)
Exercise - Indo - (n=5)
L-Name/INDO (n=4)
Exercise - Lname/Indo (n=10)

B)

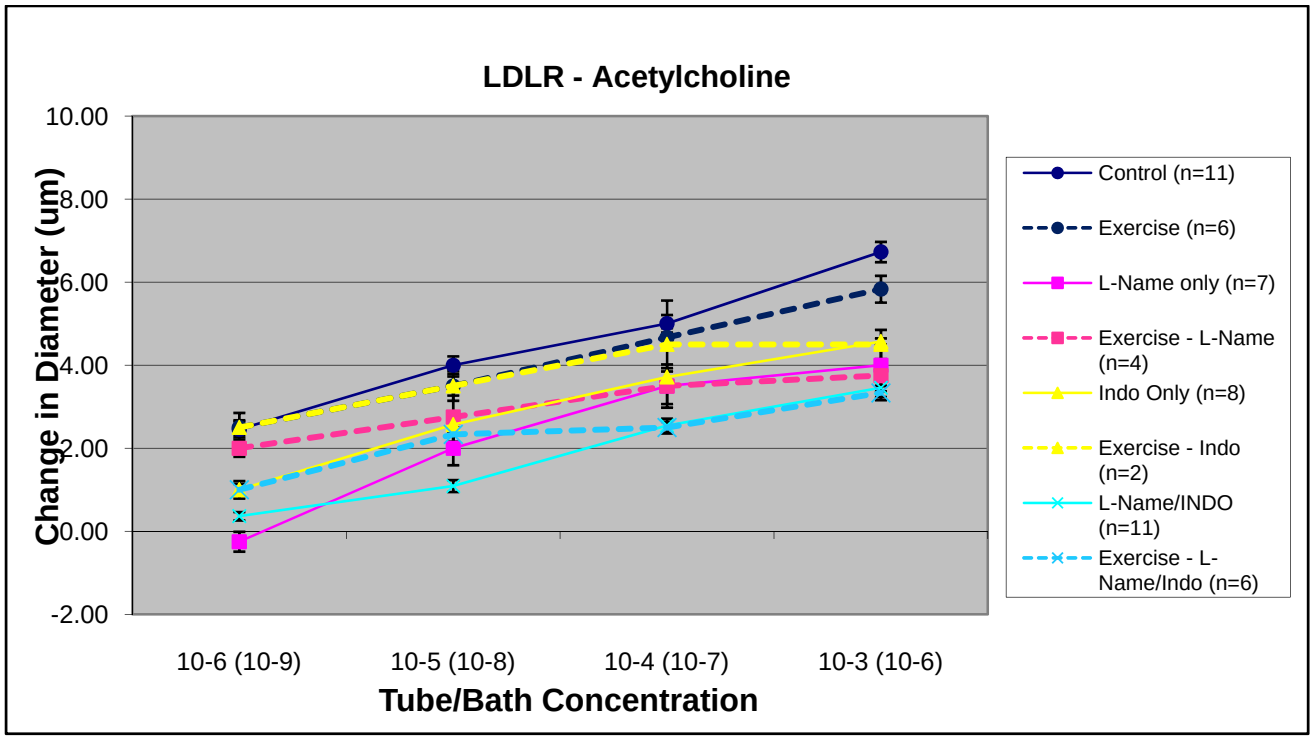
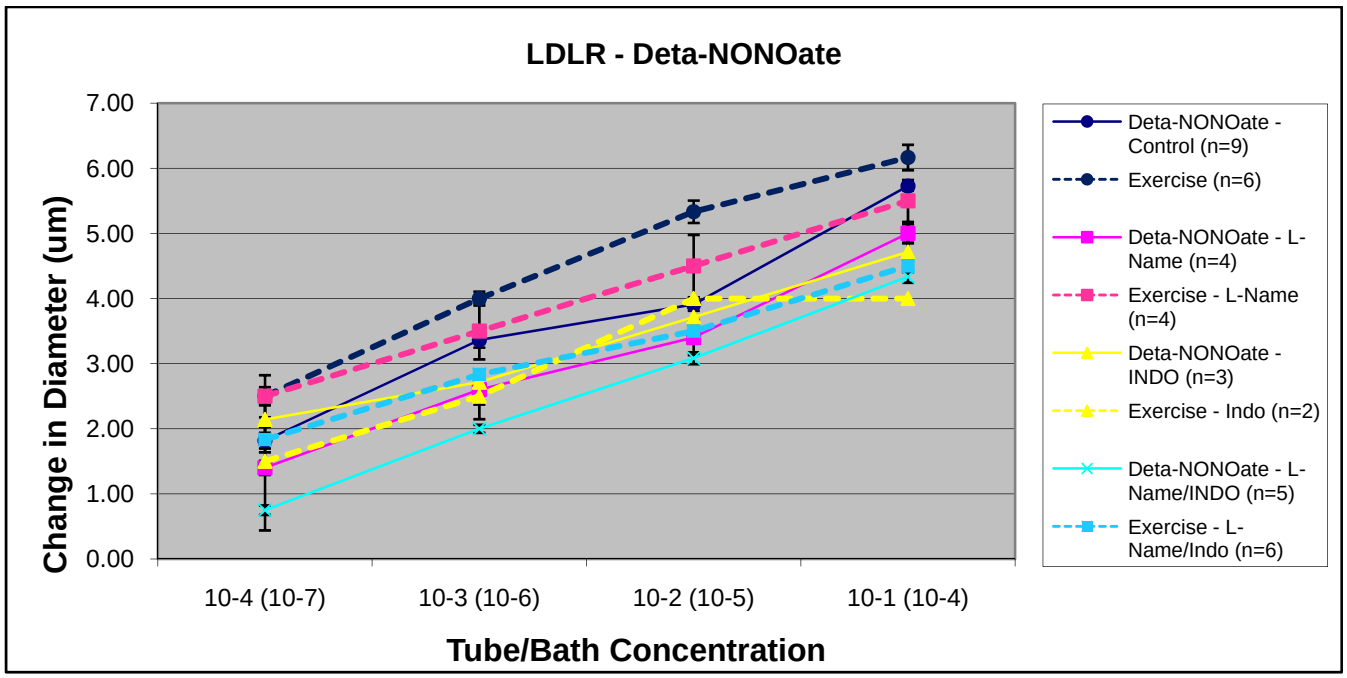

LDLR - Acetylcholine
Change in Diameter (um)
10.00
8.00
6.00
4.00
2.00
0.00
-2.00
10-6 (10-9)
10-5 (10-8)
10-4 (10-7)
10-3 (10-6)
Tube/Bath Concentration
Control (n=11)
Exercise (n=6)
L-Name only (n=7)
Exercise - L-Name (n=4)
Indo Only (n=8)
Exercise - Indo (n=2)
L-Name/INDO (n=11)
Exercise - L-Name/Indo (n=6)

Figure 5

C)


LDLR - Deta-NONOate
Change in Diameter (um)
7.00
6.00
5.00
4.00
3.00
2.00
1.00
0.00
10-4 (10-7)
10-3 (10-6)
10-2 (10-5)
10-1 (10-4)
Tube/Bath Concentration
Deta-NONOate - Control (n=9)
Exercise (n=6)
Deta-NONOate - L-Name (n=4)
Exercise - L-Name (n=4)
Deta-NONOate - INDO (n=3)
Exercise - Indo (n=2)
Deta-NONOate - L-Name/INDO (n=5)
Exercise - L-Name/Indo (n=6)

D)

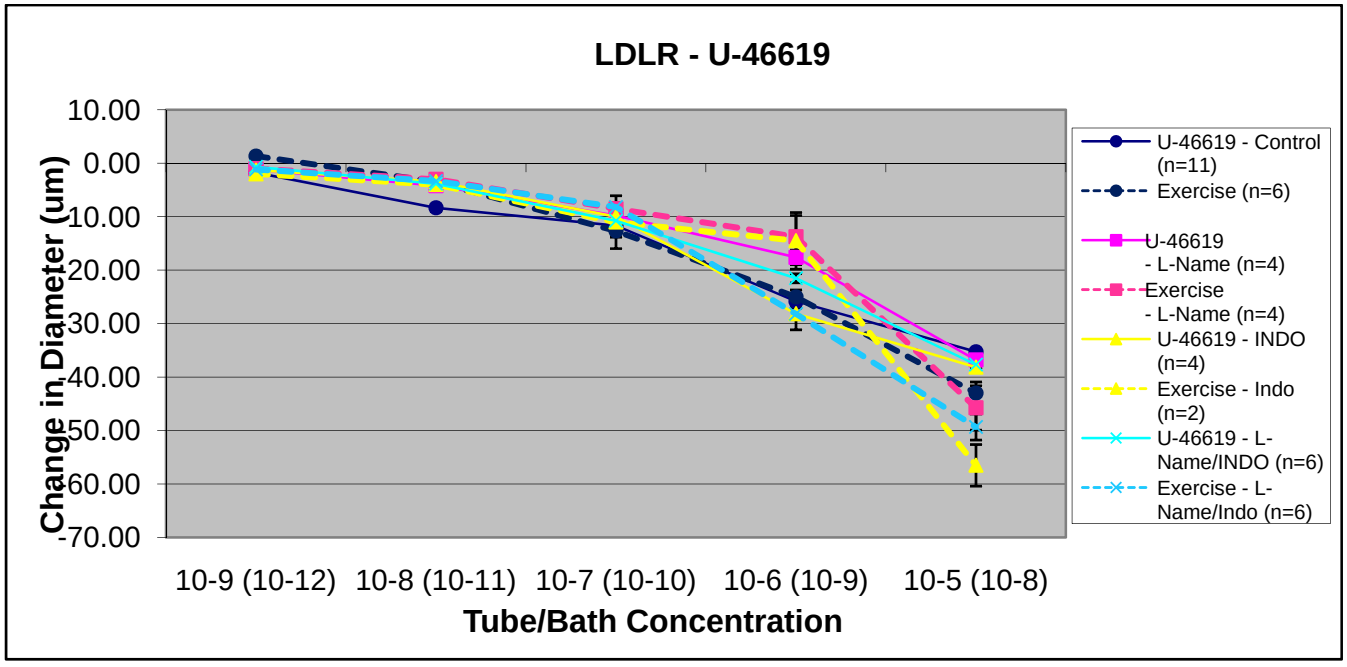
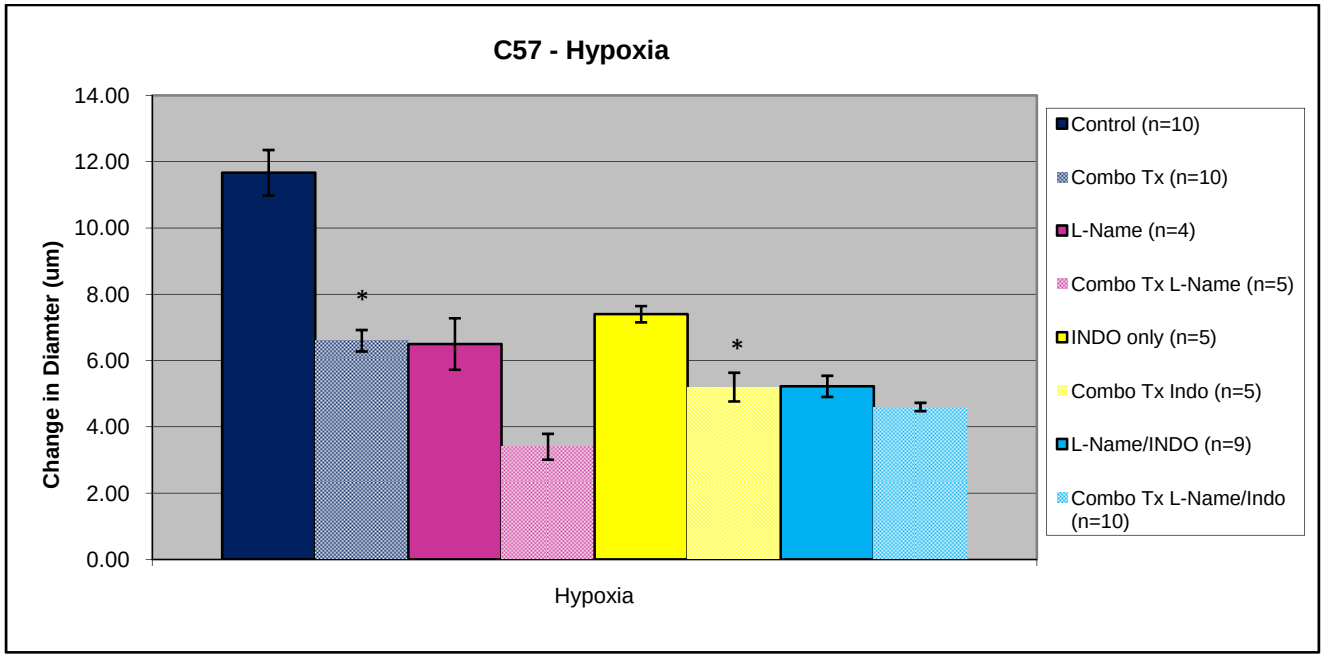

LDLR - U-46619
Change in Diameter (um)
10.00
0.00
-10.00
-20.00
-30.00
-40.00
-50.00
-60.00
-70.00
10-9 (10-12)
10-8 (10-11)
10-7 (10-10)
10-6 (10-9)
10-5 (10-8)
Tube/Bath Concentration
U-46619 - Control (n=11)
Exercise (n=6)
U-46619 - L-Name (n=4)
Exercise - L-Name (n=4)
U-46619 - INDO (n=4)
Exercise - Indo (n=2)
U-46619 - L-Name/INDO (n=6)
Exercise - L-Name/Indo (n=6)

Figure 5

A)


C57 - Hypoxia
14.00
12.00
10.00
8.00
6.00
4.00
2.00
0.00
Change in Diamter (um)
Hypoxia
Control (n=10)
Combo Tx (n=10)
L-Name (n=4)
Combo Tx L-Name (n=5)
INDO only (n=5)
Combo Tx Indo (n=5)
L-Name/INDO (n=9)
Combo Tx L-Name/Indo (n=10)

B)

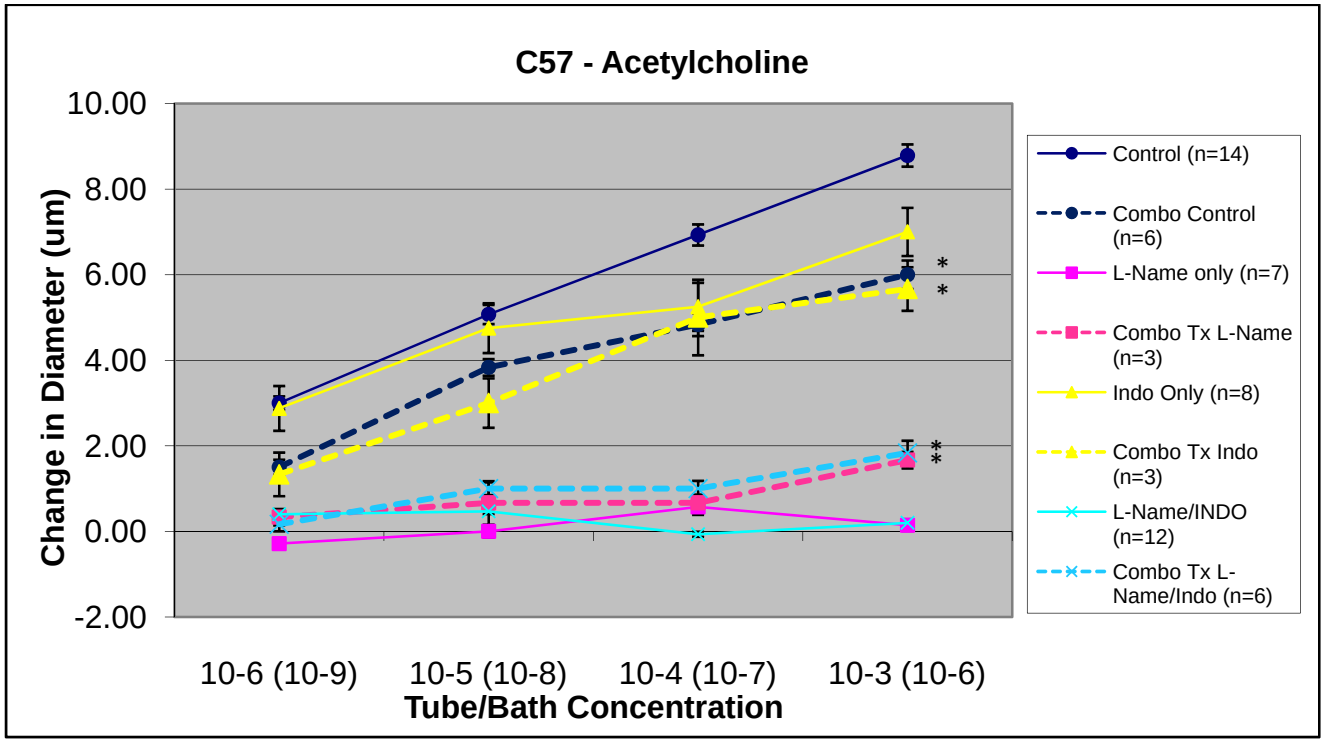
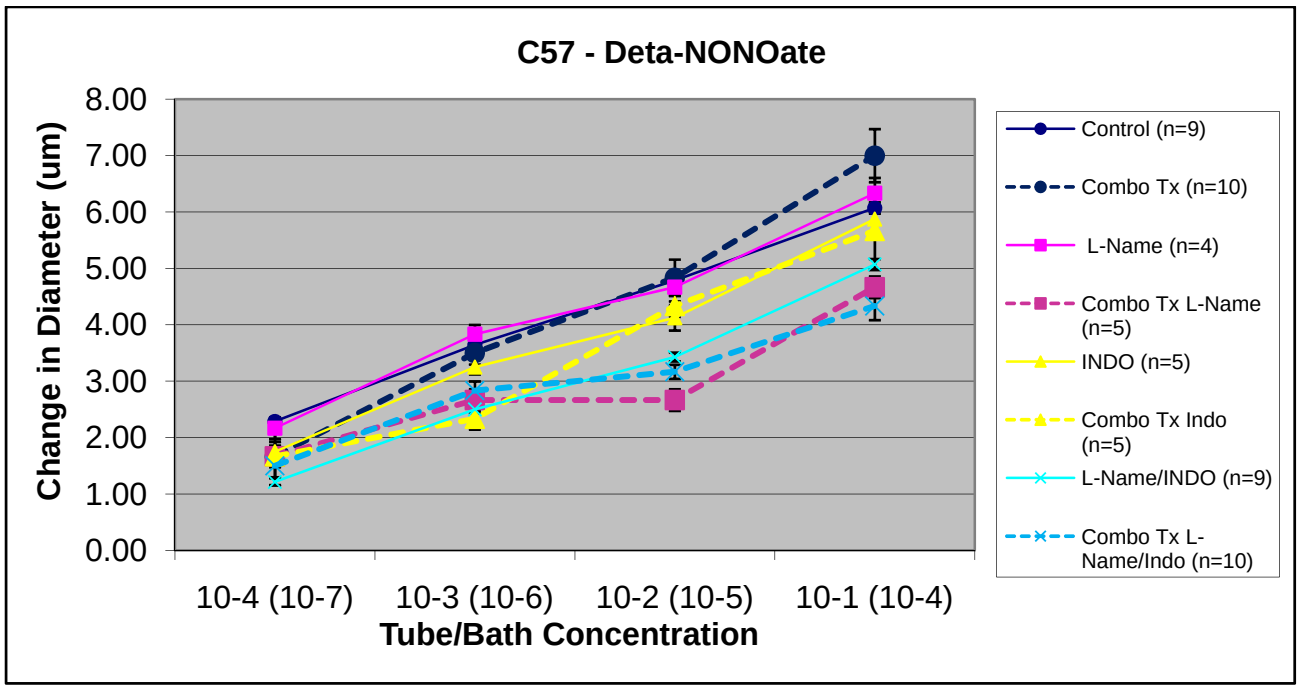

C57 - Acetylcholine
10.00
8.00
6.00
4.00
2.00
0.00
-2.00
Change in Diameter (um)
10-6 (10-9)
10-5 (10-8)
10-4 (10-7)
10-3 (10-6)
Tube/Bath Concentration
Control (n=14)
Combo Control (n=6)
L-Name only (n=7)
Combo Tx L-Name (n=3)
Indo Only (n=8)
Combo Tx Indo (n=3)
L-Name/INDO (n=12)
Combo Tx L-Name/Indo (n=6)

Figure 6

C)


C57 - Deta-NONOate
Change in Diameter (um)
8.00
7.00
6.00
5.00
4.00
3.00
2.00
1.00
0.00
10-4 (10-7)
10-3 (10-6)
10-2 (10-5)
10-1 (10-4)
Tube/Bath Concentration
Control (n=9)
Combo Tx (n=10)
L-Name (n=4)
Combo Tx L-Name (n=5)
INDO (n=5)
Combo Tx Indo (n=5)
L-Name/INDO (n=9)
Combo Tx L-Name/Indo (n=10)

D)

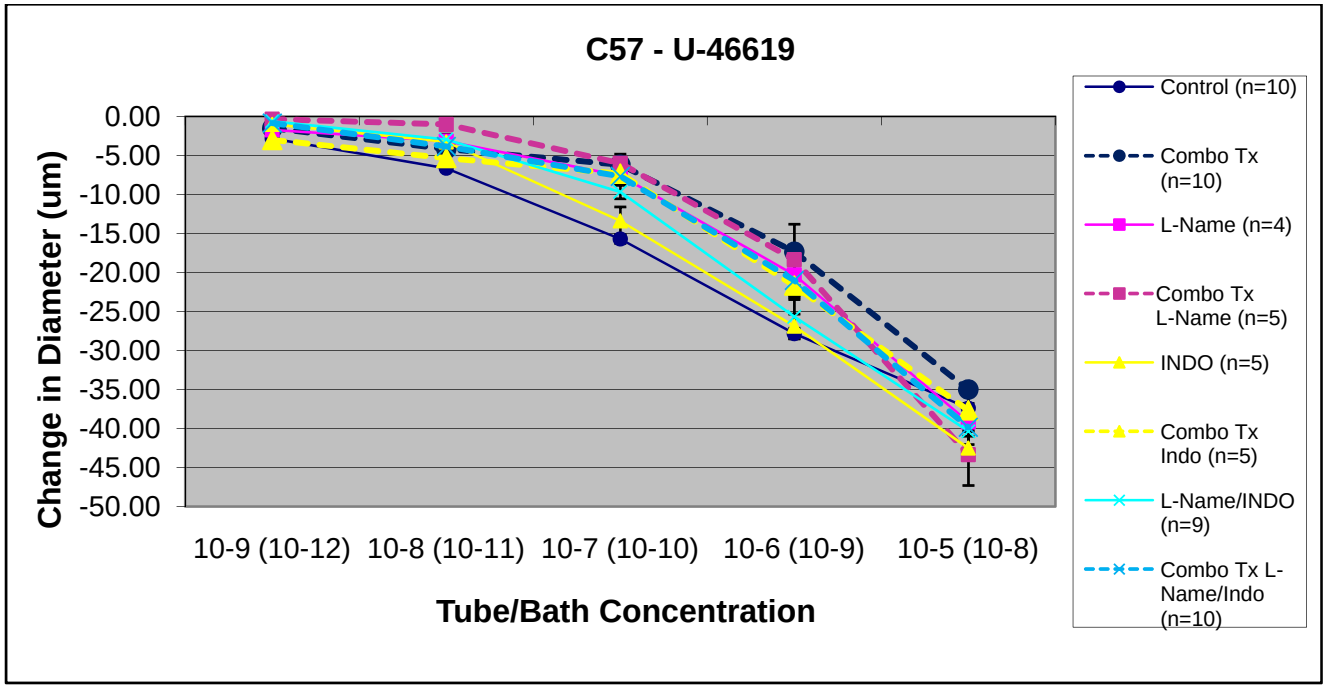
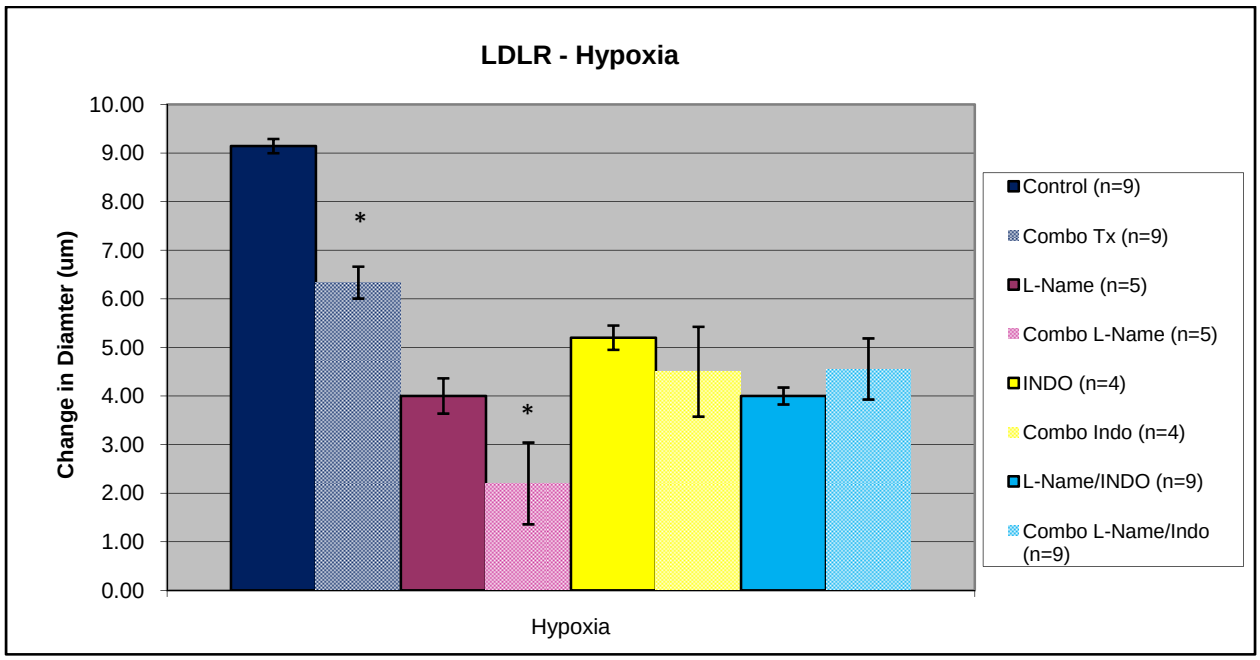

C57 - U-46619
Change in Diameter (um)
0.00
-5.00
-10.00
-15.00
-20.00
-25.00
-30.00
-35.00
-40.00
-45.00
-50.00
10-9 (10-12)
10-8 (10-11)
10-7 (10-10)
10-6 (10-9)
10-5 (10-8)
Tube/Bath Concentration
Control (n=10)
Combo Tx (n=10)
L-Name (n=4)
Combo Tx L-Name (n=5)
INDO (n=5)
Combo Tx Indo (n=5)
L-Name/INDO (n=9)
Combo Tx L-Name/Indo (n=10)

Figure 6

A)


LDLR - Hypoxia
Change in Diamter (um)
10.00
9.00
8.00
7.00
6.00
5.00
4.00
3.00
2.00
1.00
0.00
*
*
Hypoxia
Control (n=9)
Combo Tx (n=9)
L-Name (n=5)
Combo L-Name (n=5)
INDO (n=4)
Combo Indo (n=4)
L-Name/INDO (n=9)
Combo L-Name/Indo (n=9)

B)

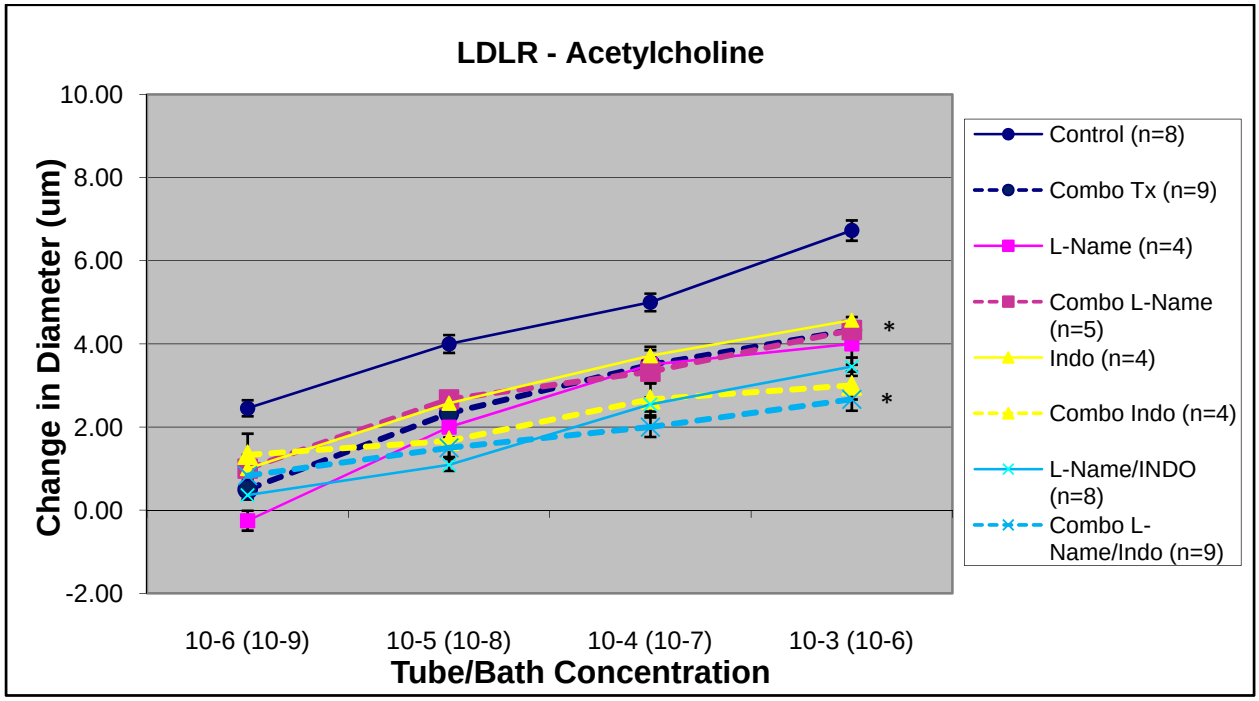
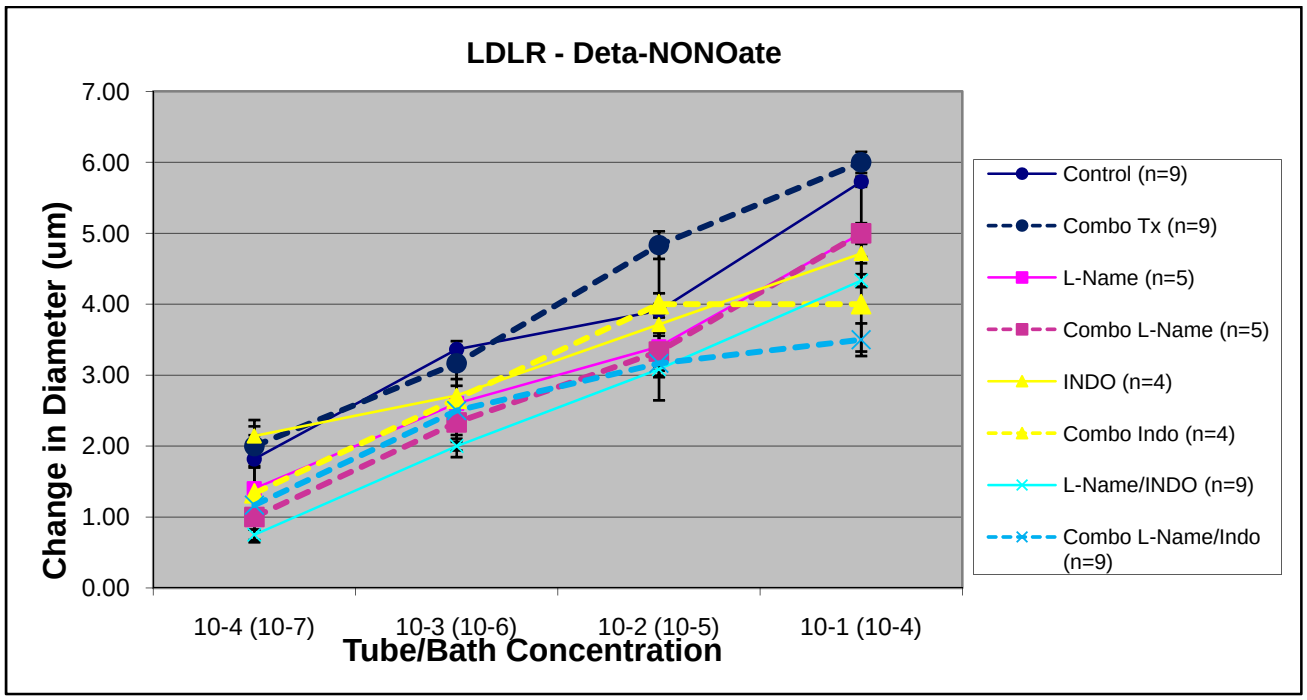

LDLR - Acetylcholine
Change in Diameter (um)
10.00
8.00
6.00
4.00
2.00
0.00
-2.00
*
*
10-6 (10-9)
10-5 (10-8)
10-4 (10-7)
10-3 (10-6)
Tube/Bath Concentration
Control (n=8)
Combo Tx (n=9)
L-Name (n=4)
Combo L-Name (n=5)
Indo (n=4)
Combo Indo (n=4)
L-Name/INDO (n=8)
Combo L-Name/Indo (n=9)

Figure 7

C)


LDLR - Deta-NONOate
Change in Diameter (um)
7.00
6.00
5.00
4.00
3.00
2.00
1.00
0.00
10-4 (10-7)
10-3 (10-6)
10-2 (10-5)
10-1 (10-4)
Tube/Bath Concentration
Control (n=9)
Combo Tx (n=9)
L-Name (n=5)
Combo L-Name (n=5)
INDO (n=4)
Combo Indo (n=4)
L-Name/INDO (n=9)
Combo L-Name/Indo (n=9)

D)

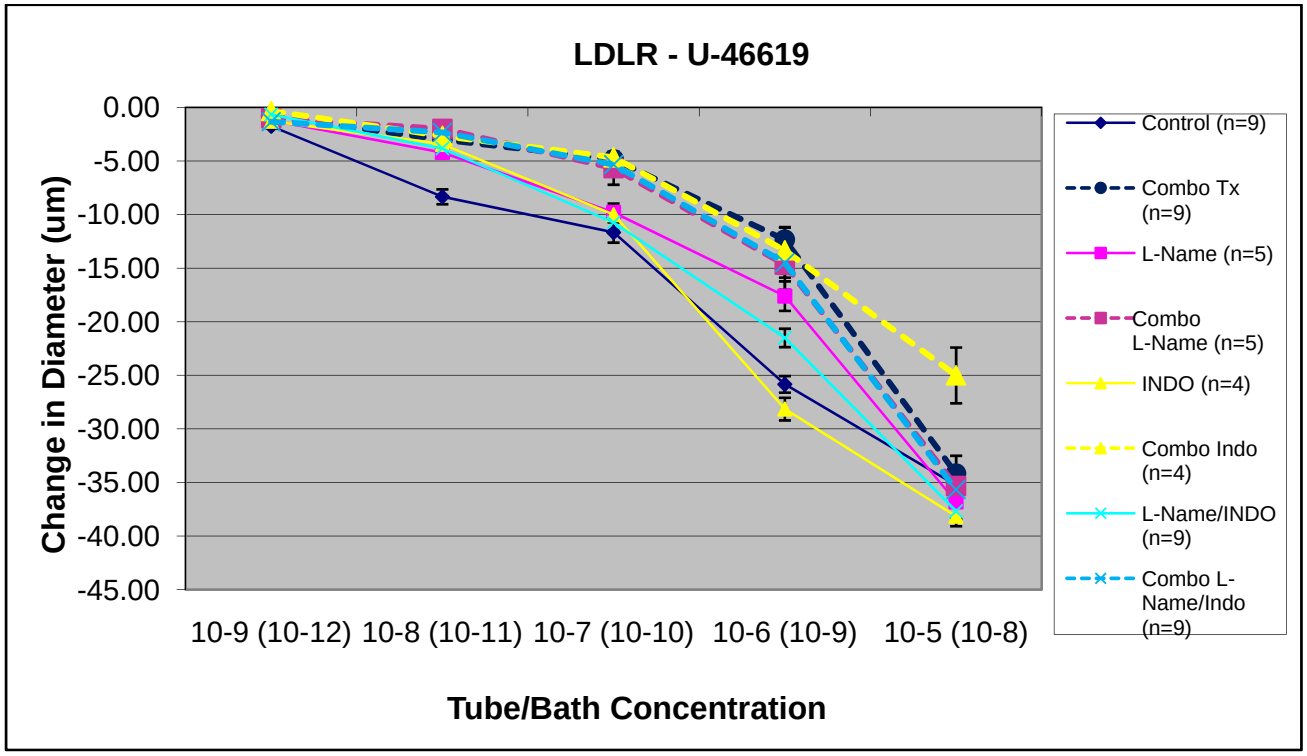

LDLR - U-46619
Change in Diameter (um)
0.00
-5.00
-10.00
-15.00
-20.00
-25.00
-30.00
-35.00
-40.00
-45.00
10-9 (10-12)
10-8 (10-11)
10-7 (10-10)
10-6 (10-9)
10-5 (10-8)
Tube/Bath Concentration
Control (n=9)
Combo Tx (n=9)
L-Name (n=5)
Combo L-Name (n=5)
INDO (n=4)
Combo Indo (n=4)
L-Name/INDO (n=9)
Combo L-Name/Indo (n=9)

Figure 7

Representative Figures

A) Representative Figures

- eNOS

- GAPDH

B) Densitometry Data

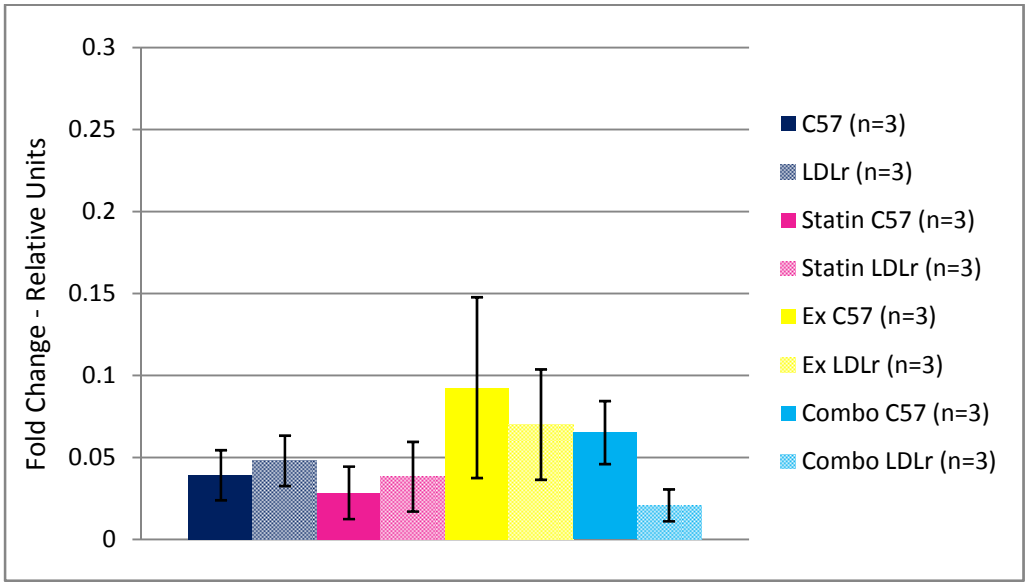


Figure 8

DISCUSSION

The purpose of this study was to investigate the condition of hypercholesterolemia and how it relates to adaptations to vascular reactivity and the mechanisms behind endothelium dependent dilation. Hypercholesterolemia has been associated with endothelial cell dysfunction at all levels of vasculature, including the microcirculation (1; 3; 6; 14; 16; 36; 43). These reports have also described impairments to vascular reactivity compared to normocholesterolemic patients (2; 5; 8; 15; 24; 34).These alterations and attenuated responses have been implicated in stemming from elevations in pro-inflammatory and oxidant stress markers, while NO bioavailability is severely blunted due to reductions in production or oxidative scavenge (3-5; 8; 10; 17-20; 24; 30; 36; 38-42). The investigation into these hypercholesterolemic consequences as they relate to vascular reactivity and the mechanisms by which they occur are currently under evaluation.

The first study, *Differential Impact of Familial Hypercholesterolemia and Combined Hyperlipidemia on Vascular Wall and Network Remodeling in Mice*, examined the differences and temporal progression of microvascular remodeling within conditions of hypercholesterolemia and hyperlipidemia. Additionally, this study compared and evaluated the mechanical alterations with plasma inflammatory biomarkers to observe the global or systemic outcomes within the three models control, familial hypercholesterolemia (FH), and familial combined hyperlipidemia (FCH). While a number of groups have evaluated the mechanistic alterations associated with dyslipidemia, these data indicate that the net modifications within dyslipidemia is not due solely to mechanistic variations, but to complicated mechanical alterations as well (28; 31; 36; 43). Our results indicate that while there is an extensive remodeling associated within the condition dyslipidemia, the specifics of the remodeling

between the two strains differs. Within the FH condition, the remodeling was within the resistance arterial wall, thereby increasing wall stiffness; additionally, cholesterol severity and inflammatory markers of cell attraction and adhesion (MCP-1) can be implicated in the early onset increase in wall thickness and stiffness. However, within the FCH condition, the remodeling was specific to the microvascular networks through an increase of rarefaction with a reflective increase in pro-inflammatory markers (TNF-α) of inflammation leading to significance at a later age. This increase in rarefaction or reduction in vascular density has been proposed to contribute to an increase in peripheral resistance and cause variations in oxygen delivery within the vascular network (12). While vascular nitric oxide bioavailability and PGI_2 prove to be poor discriminators of vascular remodeling within conditions of dyslipidemia, TxA_2 production is more significant and maintained at an earlier age within the FCH animals. Taken together, this study indicates that while there is significant remodeling leading to poor vascular outcomes within the condition of dyslipidemia; the mechanical remodeling differs with respect to the resulting dysfunction and temporal progression specific to the conditions of hypercholesterolemia and hyperlipidemia.

The second study, *Altered Mechanisms of Endothelium-Dependent Dilation in Skeletal Muscle Arterioles with Genetic Hypercholesterolemia*, examined the effects of hypercholesterolemia on the mechanisms of endothelium-dependent dilator responses of skeletal muscle resistance arterioles. While the progression and outcomes of the mechanical alterations differ, past research has shown that the dilator responses to shear and metabolic stimuli are overwhelmingly intact, despite diminished activity or bioavailability of endogenous NO (7; 29). While other groups have suggested that hypercholesterolemic animals show alterations in the endothelium-dependent dilator responses to acetylcholine and the metabolites of arachidonic acid

(32; 44). This study focuses on the maintenance of dilator reactivity despite use of alternative endothelium dependent pathways. Our results show that the endothelium dependent dilator reactivity is maintained, although the signaling mechanism to attain this dilation is altered. The signaling mechanism related to prostacyclin metabolism and activity is preserved, while NO bioavailability is diminished. With this reduction in NO bioavailability, 12- and 15-lipoxygenase emerge as a compensatory mechanism to sustain appropriate endothelium dependent dilation. This alteration suggests variations to the arachidonic acid metabolism in hypercholesterolemic animals.

The third study, *Increased Arachidonic Acid-Induced Thromobxane Generation Impairs Skeletal Muscle Arteriolar Dilation with Genetic Dyslipidemia*, examined the dilator reactivity to direct challenges of increased concentrations of arachidonic acid to explore the hypercholesterolemic alterations to the metabolism of arachidonic acid in production and vascular responses to the metabolites. Past research has strongly indicated a change to arachidonic acid metabolism, specifically, showing improvements to reactivity using cyclooxygenase inhibitors and animals lacking functional thromboxane receptors (27; 35). This study evaluated the production and vascular reactivity to arachidonic acid metabolism through the cyclooxygenase pathway specific to the condition of hypercholesterolemia. Our results indicate that there is a dilator impairment of hypercholesterolemic arterioles to increasing concentrations of arachidonic acid. These impairments do not stem from an alteration of prostacyclin production or activity; increases in thromboxane generation and activity, however can account for these decreases in dilator reactivity. Therefore, within the condition of hypercholesterolemia, altered mechanisms of arachidonic acid metabolism lead to a greater thromboxane generation. The amount of thromboxane then competes with the amount of

prostacyclin and lipoxygenase produced leading to decreased overall dilator reactivity in response to arachidonic acid metabolism. This shift in metabolism may be due to an increase in vascular oxidant stress.

The fourth study, *Impaired Skeletal Muscle Arterial Endotheluim-Dependent Dilation in Familial Hypercholesterolemia: Impact of Chronic Exercise and Anti-Cholesterol Therapies*, evaluated dilator reactivity and NO bioavailability after chronic ameliorative therapies. Hypercholesterolemia has emerged as a consistently manageable risk factor for CVD and past research has shown a number of vascular protective effects directly associated with a decrease in cholesterol levels, these effects are evident with natural cholesterol lowering treatments such as diet and exercise, along with pharmaceutical treatments. Additionally, many cholesterol lowering drugs have been linked to a number of anti-inflammatory and anti-oxidative pleiotropic effects that may lead to improvements in vascular reactivity (9; 11; 13; 21-23; 25; 26; 33; 37). This study focused on interventional strategies improving the vascular reactivity and NO bioavailability of the hypercholesterolemic animals. We found that the cholesterol lowering therapy did not improve the vascular reactivity of the hypercholesterolemic animals; in contrast, the treatment was damaging to the control animals. Overall, the effects of the cholesterol lowering treatments are beneficial with respect to the control group was detrimental and made no significant difference within the hypercholesterolemic animals. The exercise treatments alone were most beneficial to both groups. This treatment leads to an overall decrease in CRP, indicating a decrease in the overall inflammatory profile, much greater than the effects found in either of the other treatment groups.

In future studies of the endothelium dependent dilator vascular reactivity of hypercholesterolemia, there are a number of areas which warrant further investigation. Initially,

many groups have identified a decrease in the bioavailability of NO; the signaling mechanism behind this reduction may open a number of therapeutic avenues for hypercholesterolemic patients. Additionally, to determine the alterations leading to the increase in thromboxane generation and the shift leading to an increase in vascular oxidants. With respect to the interventional strategies, there are a number of conflicting reports regarding the existence of and mechanisms behind the pleiotropic effects associated with the cholesterol lowering pharmaceuticals. Furthermore, the mechanisms leading to the detrimental effect to the vascular reactivity evident in the control group also warrants further exploration.

In conclusion, hypercholesterolemia is a modifiable risk factor associated with cardiovascular diseases, including peripheral vascular and coronary heart disease. There have been numerous studies evaluating mechanical alterations and endothelial cell dysfunction leading to the impairments associated with dyslipidemic vascular reactivity. These impairments have been correlated with decreases in NO bioavailability, alterations in arachidonic acid metabolism, increases in vascular oxidants, and a pro-inflammatory condition. These studies assessed the mechanical alterations leading to poor vascular outcomes, compensatory mechanisms to maintain endothelium dependent dilator reactivity within the hypercholesterolemic condition, alterations to arachidonic acid metabolism, and evaluated the pleiotropic effects of a number of ameliorative cholesterol-lowering therapies. These results suggest that the while extensive remodeling is evident within the progression of dyslipidemia, there are significant differences between temporal development and overall outcome of FH and FCH leading to an earlier development of wall stiffness and thickness produced in part by increases in MCP-1 within FH, while the condition of FCH leads to a later progression of microvascular rarefaction, due to an increase of apoptotic markers including TNF-α; vascular function within the

hypercholesterolemic condition is maintained via the 12- and 15-lipoxygenase pathways of arachidonic acid metabolism, despite decreased NO bioavailability; arachidonic acid metabolism via the cyclooxygenase pathway is altered, leading to a greater production of thromboxane A_2 competing with prostacyclin availability thereby reducing net dilation; and clinically relevant pharmaceutical ameliorative therapies detrimentally impact normocholesterolemic endothelium dependent dilation, while making no impact on hypercholesterolemic microvessels independent of the cholesterol lowering effects of these drugs, however, exercise or combination therapies may yield the greatest benefit in both groups, as the anti-inflammatory and anti-oxidative effects hold the greatest promise in producing beneficial outcomes for both normo- and hypercholesterolemic conditions. The mechanistic and mechanical outcomes of these interventions are not well understood and require further evaluation within the realms of inflammation, oxidative stress, and vascular reactivity within the normo- and hypercholesterolemic conditions to provide the most beneficial effects.

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123 STURM ST. • CLARKSBURG, WV 26301 •
PHONE 304-624-5669 • E-MAIL PSTAPLETON@HSC.WVU.EDU

PHOEBE ADAMS STAPLETON, MSED, ATC

EDUCATION/HONORS

PhD Candidate July 2009-July 2010

PhD Student 2004-July 2009

- West Virginia University, Morgantown, WV
- Division of Exercise Physiology, School of Medicine
- Mentor: Jefferson C. Frisbee, PhD

MSEd Kinesiology 2002-2004

- Southern Illinois University Edwardsville, Edwardsville, IL
- Concentrations in Exercise Physiology and Exercise Psychology
- Thesis title: The Prevalence of Body Image Concerns Leading to Cosmetic Surgery in Female Intercollegiate Athletes (unpublished)

BS Biology and Athletic Training 1999-2002

- State University of New York College at Cortland, Cortland, NY
- Honors:

Kappa Delta Pi Induction, Educational Honor Society 2001

Beta Beta Beta Induction, Biological Honor Society 2001

Dean's List: SUNY Cortland 1999-2000

TEACHING/MENTOR EXPERIENCE

- Teaching Experience

- Exercise Physiology 2 (EXPH 567) Spring 2010

- Kinesiology (EXPH 364) Spring 2010

- Exercise Physiology 1 (EXPH 365) Spring 2010

- Human Function Small Groups (CCMD 730) Fall 2009

- Therapeutic Modalities, Independent Study Spring 2003

- Laboratory Assistant for Anatomy and Physiology 2001-2002

- Laboratory Assistant for Introduction to Biology 2000-2002

- Mentor/Supervision Assignments

  - Keli Keener, Athletic Training Student Fall 2002 – Spring 2004

  - Amul Bhalodi, Ind. Study/Research Exp. Spring 2005-Summer 2006

  - Hanna Lawther, Independent Study/Research Experience Spring 2006

  - Katie McGregor, WVU INBRE Program Summer 2007

  - Stavros Astas, Research Rotation Spring – Summer 2009

  - Jordan Beckett, WVU INBRE Program Summer 2009-2010

  - Paula Prentice, Basic Science Research Rotation Fall 2009/Spring 2010

  - Val Minarchick, Research Rotation (CCMD 797) Spring 2010

  - Catherine Kinzer, Research Rotation (CCMD 797) Spring 2010

  - Sara Fournier, Research Rotation (CCMD 797) Spring 2010

  - Joshua Butcher, Research Rotation (CCMD 797) Spring 2010

PUBLICATIONS/PRESENTATIONS

Peer Reviewed Publications

1. **Stapleton PA**, Goodwill AG, James ME, Frisbee JC. Altered mechanisms of endothelium-dependent dilation in skeletal muscle arterioles with genetic hypercholesterolemia. Am J Physiol Regul Intergr Comp Physiol., Sept; 293(3): R1110-9, 2007.
2. Goodwill AG, **Stapleton PA**, James ME, d'Audiffret AC, Frisbee JC. Increased arachidonic acid-induced thromboxane generation impairs skeletal muscle arteriolar dilation with genetic dyslipidemia. Microcirculation. Oct; 15(7): 621-31, 2008.
3. **Stapleton PA**, James ME, Goodwill AG, Frisbee JC. Obesity and Vascular Dysfunction. Pathophysiology. Aug; 15(2): 79-89, 2008.

4. Goodwill AG, Frisbee SJ, **Stapleton PA**, James ME and Frisbee JC. Impact of chronic anti-cholesterol therapy on development of microvascular rarefaction in the metabolic syndrome. Microcirculation. Aug 4:1-18, 2009.
5. **Stapleton PA,** Goodwill AG, James ME, d'Audiffret AC and Frisbee JC. Differential Impact of Familial Hypercholesterolemia and Combined Hypercholesterolemia on Vascular Wall and Network Remodeling in Mice. Microcirculation. Jan; 17(1):47-58, 2010.
6. d'Audiffret AC, Frisbee SJ, **Stapleton PA**, Goodwill AG, Isingrini E, Frisbee JC. Depressive behavior and vascular dysfunction: a link between clinical depression and vascular disease? J Appl Physiol. May; 108(5):1041-51. 2010.
7. Frisbee JC, Goodwill AG, **Stapleton PA**, Frisbee SJ, d'Audiffret AC. Aspirin Resistance with Genetic Dyslipidemia: Contribution of Vascular Thromboxane Generation. Physiol Genomics. 2010 Jun 8. [Epub ahead of print].
8. **Stapleton PA**, Goodwill AG, James ME, Brock RW, Frisbee JC. Hypercholesterolemia and Microvascular Dysfunction: Interventional Strategies. Journal of Inflammation (in review).

Oral Presentations

1. **Stapleton PA**, d'Audiffret AC, Frisbee SJ, Frisbee JC. Correlation Between Peripheral Vascular Function, Markers of Inflammation, and Depression in Human Subjects; Federation of American Society for Experimental Biology. (2009)

Abstracts and Poster Presentations

1. **Adams P**, Lox CL, Pawlow L, Covington NK, Butki BD, Gordon PM. Body Image Concerns and Cosmetic Surgery in Female Intercollegiate Athletes; American College of Sports Medicine. (2005)
2. **Adams P**, Nader GA, Visich PS, Pistilli EE, Gutmann L, Lilly C, Hoffman EP, Gordon PM. Acute Apoptotic Transcriptional Responses to Resistance Exercise Training; *FASEB J.* 2006 20:A398 (2006)
3. Nader GA, **Adams P**, Pistilli E, Guttman L, Gordan PM, Hoffman EP. Skeletal muscle remodeling during hypertrophy involves the coordinated expression of growth and atrophy genes; *FASEB J.* 20:A392 (2006)
4. **Stapleton PA**, Goodwill AG, James ME, Frisbee JC. Altered mechanisms of endothelium-dependent dilation with dyslipidemia in skeletal muscle arterioles with genetic hyperlipidemia. WVU Physiology, Julie Betschart Research Symposium. (2008)
5. Mahmoud OM, **Stapleton PA**, Frisbee JC, Mukdadi OM Noninvasive Measurement of Brachial Wall Mechanics During Flow-Mediated Vasodilation Using 2D Ultrasound Strain Tensor Imaging;

ASME Summer Bioengineering Conference. (2008)

6. Frisbee J, Goodwill AG, **Stapleton PA**, James ME, Bryner RW, Frisbee SJ. Metabolic Syndrome and Microvascular Rarefraction: Contributions from Nitric Oxide and Inflammation; 25th Conference of the European Society for Microcirculation. (2008)
7. **Stapleton PA**, d'Audiffret AC, Frisbee SJ, Frisbee JC. Correlation Between Peripheral Vascular Function, Markers of Inflammation, and Depression in Human Subjects; FASEB J. 2009 23:795.3. (2009)
8. Goodwill AG, **Stapleton PA**, James ME, d'Audiffret AC, Frisbee JC. Increased arachidonic acid-induced thromboxane generation impairs skeletal muscle arteriolar dilation with genetic dyslipidemia. FASEB J. 23:766.3. (2009)
9. **Stapleton PA**, Goodwill AG, Morrisette MR, James ME, Frisbee JC. Impaired Arteriolar Dilation in a Mouse Model of Familial Hypercholesterolemia: Impact of Chronic Exercise and Anti-Cholesterol Therapy**.** FASEB J. 24:593.5 (2010)
10. Beckett JAL, James ME, **Stapleton PA**, Goodwill AG, d'Audiffret AC, Frisbee JC**.** Insulin Resistance-Independent Impairments to Arterial Endothelial Function with Depressive Symptoms in Mice FASEB J. 24:1044.3 (2010)
11. Goodwill AG, **Stapleton PA**, Frisbee SJ, James ME, d'Audiffret AC, Frisbee JC**.** Increased Vascular Generation of Thromboxane A_2: an Initiating Condition for Microvascular Rarefaction in Obese Zucker Rats?
    FASEB J. 2010 24:774.19 (2010)
12. Goodwill AG, **Stapleton PA**, Frisbee SJ, Frisbee JC. Obesity-Induced Increased Vascular Thromboxane A_2 Generation an Initiating Stimulus for Microvascular Rarefaction? Microcirculation World Congress, 2010.
13. Frisbee J, Goodwill AG, **Stapleton PA**, Frisbee SJ, d'Audiffret AC. Aspirin Resistance with Genetic Dyslipidemia: Contribution of Vascular Thromboxane Generation. Microcirculation World Congress, 2010.

PROFESSIONAL MEMBERSHIPS

- The Microcirculation Society 2008-Present
- American College of Sports Medicine 2004-Present
- National Athletic Trainers' Association 2001-Present

REFERENCES

- Jefferson C. Frisbee, PhD
  Associate Professor of Physiology and Pharmacology
  Center for Cardiovascular and Respiratory Sciences

West Virginia University, Morgantown, WV 26506
(304) 293-6527

- Matthew A. Boegehold, PhD
  Director of the Center for Cardiovascular and Respiratory Sciences
  Professor of Physiology and Pharmacology
  West Virginia University, Morgantown, WV 26506
  (304) 293-5240
- Stephen Alway, PhD
  Professor and Chair Division of Exercise Physiology
  Director of Laboratory of Muscle Biology
  West Virginia University, Morgantown, WV 26506
  (304) 293-0772